How Lactobacilli dominate the vaginal microbiota, thereby protecting against polymicrobial communities and other vaginal infections

by

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A dissertation submitted to the Johns Hopkins University in conformity with the requirements for the degree of Doctor of Philosophy

Baltimore, Maryland

December, 2016

Abstract

Lactobacilli colonize the human vagina and acidify it with lactic acid. In this highly acidic environment Lactobacilli manage to achieve a sparse, but near monomicrobial community, in which very few other species of bacteria are present. Despite the acidity of the vaginal environment, I have found that vaginal Lactobacilli grow optimally at a near neutral pH in cervicovaginal fluid (CVF) or culture media. Even when directly exposed to the acidity of the vagina, however, I observed *ex vivo* that the Lactobacilli are still growing and producing lactic acid at a slow rate. Women who do not have a monomicrobial community of Lactobacilli, have a polymicrobial community made up of many different gram-negative bacteria. Using *ex vivo* conditions I determined that vaginal lactic acid was sufficient to inactivate bacteria from the polymicrobial vaginal communities, precluding the necessity of peroxide production or bacteriocins, which have been previously suggested. Therefore, by maintaining high vaginal acidity, even at a slowed growth rate, Lactobacilli can protect the vagina against other communities of bacteria. Mild acidification beyond the stable pH attained by Lactobacilli *in vivo*, however, inactivated the Lactobacilli themselves. For the vaginal pH to remain stable and above what is fatal to the Lactobacilli, it requires a model in which lactic acid is lost from the vagina at the same rate as which the bacteria produce it, and where the rate the bacteria grow and adhere to the epithelium matches the rate of vaginal epithelial cell shedding. The majority of women worldwide have a polymicrobial community of vaginal bacteria, putting them at a higher risk for the acquisition and transmission of sexually transmitted infections, and poor birth outcome. For unknown reasons this risk also extends to women with specific *Lactobacilli*

spp. By measuring the vaginal acidity across different community state types I found that the higher risk Lactobacilli community (CST III-INERS) was correlated with less vaginal acidity. Therefore, the difference in risk between different bacterial communities is likely due to a difference in vaginal acidity. Vaginal acidity may inactivate pathogens outright, or protect a Lactobacilli dominated community by inactivating bacteria from polymicrobial vaginal communities. Even women with high vaginal acidity, however, have been observed to occasionally transition to a polymicrobial community. This suggests that other mechanisms must exist to determine which bacterial communities can remain in the vagina. Few studies have attempted to determine if the mucosal immune system plays a role in maintenance of vaginal bacteria communities, and of these studies none of have reported on host-antibody coating of vaginal bacteria, despite the important role it plays in the maintenance of gut bacteria. Using both microscopy and flow cytometry approaches I report antibody coating of vaginal Lactobacilli. Each method revealed extensive coating with IgG and IgA. A subset of the cultured bacteria from a CVF sample could be recoated with host antibodies from the CVF supernatant, suggesting some antibody specificity, and a potential role for the mucosal immune system in the regulation of vaginal bacterial communities.

Acknowledgements

I am very grateful to my thesis advisor Richard Cone for accepting me into his lab and for his overwhelming encouragement and support both scientifically and otherwise. I've greatly enjoyed our relationship over the last three years and could not have asked for a better advisor.

I would also like to thank my thesis committee, Doug Barrick and Jocelyn DiRuggerio, for taking an interest in my work and whose pointed questions were helpful in shaping the direction of this project.

I want to thank Tom Moench a member of our lab whose shrewd approach to science was always appreciated to help plan good experiments. I've greatly enjoyed my discussions with him over the last few years. I want to thank the other members of the Cone lab past and present—Bizzy O'Hanlon for being my initial mentor in the lab, Tim Hoen for managing the lab and regaling me with tales about life, Ying-Ying Wang for her contributions to this work, Derek Cerchione for his contributions to this work and all the wonderful undergraduate students I had the pleasure of working with.

I would like to thank my secondary reader and collaborator Laura Ensign-Hodges for reading this thesis as well as for her enthusiastic support and encouragement. I also thank her and Thuy Hoang for helping me gain access to high Nugent CVF samples. I've enjoyed collaborating with them both.

I thank my friends both from this program and elsewhere, for their scientific feedback and everything else—especially my classmate Rachel Neiderer.

Finally, I thank my family who have always provided an understanding and supportive environment, while encouraging me to accomplish my goals.

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Introduction: Lactobacilli dominated and polymicrobial vaginal communities

The discovery of vaginal Lactobacilli and polymicrobial vaginal bacterial communities

In 1892 German gynecologist Albert Döderlein published his discovery of gram-positive rod-shaped lactic acid producing bacteria in the vagina (Döderlein, 1892). He named the bacteria "Döderlein's bacillus" and predicted that the production of lactic acid was crucial for protecting the vagina against bacterial infections, such as the "abnormal" bacteria observed in women who did not have "Döderlein's bacillus" (Rogosa and Sharpe, 1960). "Döderlein's bacillus" was soon determined to be *Lactobacilli* spp. and today the "abnormal" gram-positive bacteria can be clinically described as Bacterial Vaginosis (BV).

Neither of these vaginal bacterial community types are present in prepubescent girls (except for a transient colonization in newborns elicited by maternal estrogen), or post-menopausal women (Cruickshank, 1934; Cruickshank and Sharman, 1934; Farage and Maibach, 2006). Instead, during these periods of low estrogen vaginal bacterial communities consist of a low concentration of various gram-positive bacteria. Vaginal communities dominated by *Lactobacilli* spp. and polymicrobial communities, arise after puberty due to estrogen-initiated deposition of glycogen in the vaginal epithelium. Despite the abundant supply of glucose due to host amylases depolymerizing glycogen (Spear et al., 2014), it is remarkable that some women have sparse *Lactobacilli* spp. dominated communities that are far less dense than the polymicrobial communities.

The prevalence of polymicrobial vaginal bacterial communities

For the past 25 years, vaginal communities have been characterized with Nugent scoring based on bacterial morphotypes (Nugent et al., 1991). A meta-analysis of BV prevalence studies reported that the prevalence of BV in American sample sets ranged 19.3-29.2% (Kenyon et al., 2013). Importantly, white women were less likely to be diagnosed as having BV (14-24.7%) compared to black women (25.8-51.4%), with Hispanic women tending to fall in between. Prevalence studies in other countries indicate BV prevalence correlates with the fraction of the population with African descent. Attempts to remove socioeconomic factors as confounding variables do not diminish the higher risk of having BV for being black (Koumans et al., 2007; Ness et al., 2003). However, the methodology involved in correcting for socioeconomic factors remains insufficient (Williams et al., 2010), and until better methodologies are put forward, care should be taken to avoid identifying being black as a causal agent of BV.

Although roughly one-third of women were shown to have BV in the United States by Nugent scoring, only another one-third of women were predicted to have a vaginal bacterial community dominated by *Lactobacilli* spp. (Koumans et al., 2007). The remaining women are categorized as "intermediate" under Nugent scoring, and traditionally the label of "BV" is not extended to these women. The utility of distinguishing between "BV" and "intermediate" seems less important in the light of 16S community sequencing, which has not revealed bacterial community types that are specific to either "BV" or "intermediate" and not the other (Ravel et al., 2011). Instead, both types can be characterized as polymicrobial communities lacking a predominant *Lactobacilli* spp. Therefore, in this thesis polymicrobial communities will

refer to both “BV” and “intermediate” diagnoses by Nugent score, unless otherwise specified. Combining the prevalence of “BV” with the prevalence of “intermediate” reveals that roughly two-thirds of women have a polymicrobial vaginal bacterial community, and only a minority have lactobacillus-dominated communities.

Risks associated with a polymicrobial vaginal bacterial community

Significant risks are associated with polymicrobial vaginal communities. Although many risk studies have focused only on women with a “BV” diagnosis, the extension of inclusion to any women with a polymicrobial vaginal bacterial community has revealed similar risk, validating our choice to distinguish only between polymicrobial communities and Lactobacilli dominated communities.

The risk factors associated with a polymicrobial community fall primarily into two categories: 1) Risk of acquisition, and transmission, of sexually transmitted infections (STIs), 2) Birth complications. A meta-analysis of the risk of HIV acquisition has reported that women with BV are 60% more likely to contract HIV than other women (Atashili et al., 2008). Importantly, studies grouping together any polymicrobial community reveal an association between HIV infection and polymicrobial communities (Cohen et al., 1995; Martin et al., 1999). Women with a polymicrobial community are also more likely to transmit HIV to their male partners (Cohen et al., 2012), potentially due to increased shedding of active virus in women with BV (Sha et al., 2005). A polymicrobial community is also more likely associated with, or puts a woman at elevated risk for trichomonal, gonococcal, and chlamydial infections (Brotman et al., 2010a; Martin et al., 1999; Wiesenfeld et al., 2003).

Meta-analysis reveals that women with BV are more likely to experience miscarriage or preterm birth, especially if the BV is detected during early pregnancy (Leitich et al., 2003). More recent evidence using 16S sequencing has established that preterm birth is associated with not just BV but any polymicrobial community (DiGiulio et al., 2015; Hyman et al., 2014). There is a clear association between a polymicrobial community and pelvic inflammatory disease (PID), but a causal relationship has not yet been established (Taylor et al., 2013).

Treatment of BV remains a difficult task as is discussed further below. Without a clear treatment it remains very difficult to convincingly establish strong causal relationships between a polymicrobial community and its associated risks. With nearly two-thirds of women at an elevated risk for STI transmission and acquisition and poor birth outcome, it is paramount to find a way to treat polymicrobial communities or introduce a Lactobacilli dominated community, both to better establish causal relationships between a polymicrobial community and associated risks and likely to prevent them.

The etiology of polymicrobial vaginal bacterial communities

The etiology of a polymicrobial community is poorly understood. Many risk factors have been associated with BV prevalence including smoking (Brotman et al., 2014; Cherpes et al., 2008; Hellberg et al., 2000), douching (Brotman et al., 2008; Holzman et al., 2001; Ness et al., 2002), stress (Nansel et al., 2006) and sex practices including multiple male partners, a recent change in male partners, not using a condom or a recent female partner (Fethers et al., 2008), uncircumcised male partners or rectal sex before vaginal sex (Cherpes et al., 2008) and the use of lubricants (Brotman et al., 2010b).

Given the association of certain sex practices with BV it is possible that BV is transmissible between partners. Uncircumcised male partners of women with BV are more likely to have penile bacterial communities that include BV bacterial species (Liu et al., 2015) and one instance of suspected male to female transmission has been reported (Muzny and Schwebke, 2014). Treating male partners with antibiotics, however, has not been effective for preventing recurrent BV (Amaya-Guio et al., 2016; Mehta, 2012), suggesting that transmission from male partners does not explain many cases of recurrent BV. Amongst women who have sex with women (WSW) high concordance is observed in the vaginal bacterial community of partners (Evans et al., 2007; Marrazzo et al., 2002; Vodstrcil et al., 2014). This suggests that BV and/or Lactobacilli are being transmitted between partners. Interestingly, although these and other studies have reported female sex partners as a risk factor for BV (Koumans et al., 2007), analyzing sexual practices amongst WSW found a much higher risk of BV in WSW that used lubricants than those that did not (Marrazzo et al., 2010). This suggests that cases of concordant polymicrobial communities could be the result of shared sex practices which either cause a polymicrobial community to arise separately in each individual, or increase the chance of one partner with a polymicrobial community to transmit it to the other.

The large number of risk factors associated with a polymicrobial community despite an inability to prevent recurrent BV by a single intervention suggests that acquisition is a multivariable event. It is very likely that different women obtain their vaginal community in different ways. Furthermore, initial exposure or transition to a polymicrobial community is likely mechanistically different than recurrence. Unlike Lactobacilli which maintain both a sparse concentration of bacteria and space between individual bacteria, BV bacteria form

extensive dense biofilms. Therefore, factors that cause BV may not be relevant to transmission, but to giving a fitness advantage to residual BV bacterial biofilms.

The failure of antibiotic treatment and recurrent BV

Treating BV, especially recurrent BV remains a daunting task. Currently, the standard of care for symptomatic BV is antibiotic treatment, typically metronidazole or clindamycin (Centers for Disease Control and Prevention, 2015). Antibiotic treatment of BV has a high short-term cure rate—80-90% after one week of treatment (Larsson, 1992)—but relapse occurs frequently. Many studies have identified recurrence as early as one month after treatment (Bradshaw et al., 2006; Sobel et al., 1993), and agree that after a year the majority of women will have had a recurrent episode of BV (Boris et al., 1997; Bradshaw et al., 2006). In these studies, sexual practices, including new sexual partners were most highly associated with recurrence.

Probiotic approaches to treating BV

For nearly two decades vaginal probiotics have been explored to improve the low efficacy of antibiotics to prevent recurrent BV. Unfortunately, the majority of these approaches have had mixed results. Three large meta-analyses of vaginal probiotic trials found no conclusive evidence that using vaginal probiotics prevents recurrent bacteria (Falagas et al., 2007; Homayouni et al., 2014; Senok et al., 2009). Some trials are more successful than others, but most of these trials do not treat transition to a bacterial community dominated by *Lactobacilli* spp. as the positive outcome, instead relying on overly sensitive detection methods of any *Lactobacilli* spp., or an indirect diagnosis of BV such as Amsel's criteria.

An efficacious vaginal probiotic remains a high priority in the field given the failure of antibiotics to prevent recurrent BV. It remains unclear why vaginal probiotic attempts have failed, but it is likely that the vaginal environment and the state of the bacteria in the probiotic are not compatible. Therefore, in order to one day create a better probiotic, we need to understand the basic biology of how Lactobacilli maintain themselves, and dominate the vaginal environment.

Vaginal Lactobacilli acidify the vagina with lactic acid

Even in 1892, Döderlein predicted that vaginal lactic acid was the key to the prevention of "abnormal" bacterial communities. Experiments by our lab were the first to measure both the degree of acidity in cervicovaginal fluid (CVF) from individuals with a Lactobacilli-dominated vaginal community, and to establish that the Lactobacilli were the source of such acidity. Original measurements of vaginal pH were highly variable and failed to exclude individuals with a polymicrobial vaginal community (Cohen, 1969; Hunter and Long, 1958). A more contemporary review of vaginal pH measurements reported an average pH of 4.2 for women with a Lactobacilli dominated community (Owen and Katz, 1999). With the advent of Nugent scoring our lab was able to make pH measurements in CVF obtained from individuals with Lactobacilli dominated vaginal communities. Under standard laboratory conditions we found that the pH of CVF in these individuals was 3.74 (O'Hanlon et al., 2013). However, measuring the pH of CVF outside the vaginal environment does not take into effect alkalinizing effect of the loss of carbon dioxide on aerobic exposure. Measuring the pH of CVF under 5% CO_2 confirmed this concern, yielding an average pH of 3.48. A second study was performed,

measuring the vaginal pH directly with a vaginally inserted pH electrode and confirmed the accuracy of the in vitro method under 5% CO_2. These studies establish that the acidity of the Lactobacilli dominated vagina is significantly lower than previously thought. The acidity of the vagina is surpassed only by that of the stomach, adding intrigue to the mechanisms by which Lactobacilli themselves are able to survive and thrive.

The chemical nature of lactic acid allowed our lab to determine that vaginal acidity must be produced by the vaginal bacteria and not the host. Lactic acid is a chiral molecule, typically produced by stereoisomer specific lactate dehydrogenases to replenish NADH through the conversion of NAD^+. Although humans lack the D-lactate dehydrogenase enzyme, we found that many vaginal samples contained high concentrations of D-lactate (Boskey et al., 2001). This suggested a bacterial source for D-lactate. Moreover, culturing the vaginal samples in growth medium resulted in cultures with a similar D:L ratio to that of the original CVF, confirming that the majority of vaginal lactic acid is produced by Lactobacilli.

The lactic acid concentration of CVF is tightly inversely correlated to the pH in Lactobacilli dominated samples (O'Hanlon et al., 2013). Since the Lactobacilli are the source of the lactic acid, this suggests that vaginal pH is also determined by the bacteria, and not by the host. Corroborating this hypothesis, we found that cultures of vaginal Lactobacilli not only reach the D:L ratio of the original CVF, but the final pH directly correlates as well—more acidic cultures arise from more acidic vaginal samples (Boskey et al., 1999). Not only are the bacteria the source of vaginal acidity, but there must be differences within the strains of bacteria that cause higher or lower levels of lactic acid, and therefore pH. This is the subject of Chapter 2.

Lactic acid is a potent microbicide

Amongst women who have vaginal Lactobacilli, BV is more frequently associated with strains of Lactobacilli that do not produce H_2O_2 (Cherpes et al., 2008; Eschenbach et al., 1989). Furthermore, amongst women with a Lactobacilli dominated vaginal bacterial community, the risk of STI acquisition is lower if the Lactobacilli produce H_2O_2. This raised the plausible hypothesis that H_2O_2 produced by lactobacilli is responsible for inactivating BV associated bacteria and vaginal pathogens. Although high concentrations of H_2O_2 producing Lactobacilli were toxic to BV bacteria *in vitro* while non-H_2O_2 producing Lactobacilli were not, the effect was abolished by raising the pH (Klebanoff et al., 1991).

Using lactic acid solutions as well as cell-free CVF samples from women with Lactobacilli dominated communities, our lab demonstrated that lactic acid was important for the inactivation of BV-associated bacteria *in vitro* and not H_2O_2 (O'Hanlon et al., 2011). H_2O_2 solutions were microbicidal to BV-associated bacteria, however, a minor addition of cell-free CVF completely abolished the inactivation, likely due to the high antioxidant properties of CVF. Importantly, the microbicidal activity of lactic acid was abolished at a neutral pH, and an acidic pH without lactic acid was not nearly as effective. This suggests that the protonated form of lactic acid is important for inactivation. Aside from BV-associated bacteria, lactic acid is capable of inactivating HIV (Aldunate et al., 2013), *Chlamydia trachomatis* (Gong et al., 2014) and HSV (Conti et al., 2009). Therefore, many of the risks associated with a polymicrobial community are most likely due to the lack of lactic acid. This subject is addressed in Chapter 3.

The unexplored role of the mucosal immune system in regulating vaginal bacterial communities.

Despite extensive studies of the role of the mucosal immune system in regulating GI tract microbiota, no studies to date have examined its role in the vagina. As a first step in beginning this exploration, Chapter 4 reports that healthy, non-inflammatory vaginal lactobacilli are coated with host antibodies.

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How vaginal Lactobacilli regulate the vaginal pH

Chapter abstract

Lactobacilli colonize the human vagina and acidify it with lactic acid. What has remained unclear is how vaginal Lactobacilli regulate the pH of the vagina and whether the lactic acid they produce is sufficient for them to dominate the vaginal bacterial community. Here I report that in *ex vivo* cultures as well *in vitro* cultures of cervicovaginal fluid CVF, the growth rate of vaginal Lactobacilli slows as they approach vaginal pH. At vaginal pH the replication rate of Lactobacilli must match the rate at which these bacteria are shed from the vagina by the rapid shedding of vaginal epithelial cells. Also, at this growth rate Lactobacilli must produce lactic acid at the same rate it leaves the vagina; it is this steady state of growth rate and lactic acid production rate that determines the pH of the vagina. I find that at this acidic pH the concentration of lactic acid is a broad-acting and potent microbicide sufficient to inactivate polymicrobial communities in *ex vivo* CVF samples from individuals with Bacterial Vaginosis (BV). Lactobacilli are close to killing themselves with this lactic acid acidity: In *ex vivo* cultures, modest over-acidification, and/or modest addition of lactic acid at vaginal pH speeds the death of vaginal lactobacilli. In regulating the steady-state of vaginal pH, the permeability of the vaginal epithelium to lactic acid, and the shedding rate of vaginal epithelial cells are important host factors that likely determine the strains of lactobacilli that can maintain dominance over the microbiota of the vagina.

Background Information

Vaginal Lactobacilli determine the acidity of the vagina through the production of lactic acid

Over a century ago, Döderlein proposed that the healthy human vagina is acidified by bacteria (Döderlein, 1892). His hypothesis was based on his discovery of lactic acid producing bacteria, later identified as Lactobacilli, within the vagina that were not present in women who did not have acidic cervicovaginal fluid (CVF). Over the years the degree to which Lactobacilli were responsible for determining the vaginal acidity was contested. An alternate source of lactic acid is from epithelial cell metabolism in the hypoxic environment of the vagina. Others acknowledged the role of Lactobacilli in acidifying the vagina, but believed the presence of BV bacteria could produce alkalizing agents and raise the pH (Pybus and Onderdonk, 1999).

Our lab determined that Lactobacilli are sufficient to explain vaginal acidity. The pH of the vagina varies between women, and correlates strongly with the asymptotic pH to which vaginal Lactobacilli acidify their growth medium (Boskey et al., 1999). More convincingly, different species and strains of vaginal Lactobacilli produce different ratios of the optical isomers of lactic acid, and cultures of vaginal Lactobacilli produce D:L ratios of lactic acid that correlate strongly with the D:L ratio in the cervicovaginal fluid (CVF) from which they are obtained (Boskey et al., 2001). In particular, when strains of *Lactobacillus crispatus* dominate the vaginal microbiota, they produce more D than L isomers and the high D:L ratios strongly correlate between cultured CVF and the CVF itself (Witkin et al., 2013). In contrast, human metabolism can only produce the L isomer. Although these approaches do not rule out the vaginal epithelium as a source of lactic acid, it suggests that the amount being produced is not

enough to influence the vaginal pH compared to the amount being produced by Lactobacilli. If Lactobacilli determine the acidity of the vagina, they also must regulate it.

The pH of the vagina is stably acidic, despite *Lactobacilli* spp. preferring a neutral environment

Vaginal Lactobacilli acidify the vagina to pH 3.6 on average with a lactic acid concentration around 110 mM when they are the predominant species in the vaginal bacterial community (O'Hanlon et al., 2013). This environment is unique both within the human body, where only the stomach is more acidic, and amongst other mammals. Humans remain the only primates in which *Lactobacilli* spp. have been found to predominate the vaginal bacterial community (Stumpf et al., 2013).

Although capable of acidifying their environment, the optimal growth of non-vaginal species of Lactobacilli such as *L. delbruekii, L. acidophilus,* and *L. paracasei* occurs at near neutral pH (Adamberg, 2003). Acidification of the growth media through the production of lactic acid beyond the optimal pH slows the rate of growth in these strains. This problem has been well understood in the food industry. To maximize the concentration of bacteria, Lactobacilli starters are grown in pH-controlled bioreactors (Rault et al., 2009). By holding the pH stable the population can continue to grow at its optimal rate. Recently, we recorded vaginal pH *in vivo* with intravaginal electrodes, and found the pH to be very stable, remaining within 0.1 pH units over 90 or 45 min intervals (O'Hanlon, in submission). To achieve stable acidic conditions in the vagina, vaginal Lactobacilli must continue to grow, replicate and produce lactic acid at rates that equal the loss of bacteria and the loss of lactic acid from the

vagina. Unlike non-vaginal strains of Lactobacilli, vaginal strains might grow optimally at the low pH values observed in CVF. However, one of the only studies that looked at the effect of initial culture pH on the growth of a vaginal species of Lactobacilli used an ATCC strain of *L. gasseri* and observed a decreased growth rate at pH 4 compared to 5 or 6 (Boskey et al., 1999). Alternatively, vaginal Lactobacilli are maintaining a non-optimal environment in regards to growth.

Bacterial acid adaptations

Another possibility, is that to continue growing at a rapid enough rate, vaginal Lactobacilli might minimize their exposure to the acidity. *Helicobacter pylori* despite colonizing the human stomach is well adapted to minimize its exposure to the highly acidic stomach acid. Similar to *Lactobacilli* spp., *H. pylori* grows optimally between 5.5 and 8.5 (Morgan et al., 1987; Scott et al., 1998) and is actually inactivated if exposed to pH values below 3.5 due to a collapse of its transmembrane potential difference (Meyer-Rosberg et al., 1996). The ability of *H. pylori* to survive *in vivo* is thought to depend on its chemotactic motility to penetrate the gastric mucus layer to reach the less acidic pH near the epithelium (Schreiber et al., 2004). If vaginal Lactobacilli use a similar strategy, Lactobacilli suspended in the vaginal lumen should be dead or dying, and only those residing at a higher pH, perhaps deep within the epithelium, would grow and produce lactic acid.

Although it is possible that *Lactobacilli* spp. and *H. pylori* use similar mechanisms, there are also many differences between the two bacteria. In the case of *H. pylori* the source of the acid is the gastric epithelium, and the highest acidity is achieved away from the epithelium and

the mucin layer, likely due to viscous fingering (Bhaskar et al., 1992). Lactobacilli themselves are the source of the acid, suggesting it would be more difficult for them to avoid exposure. *H. pylori* also releases the enzyme urease into its periplasmic space. Cleavage of urea results in two molecules of the proton-acceptor NH_3 thus increasing the pH within the periplasmic space (Bode et al., 1993; Scott et al., 1998). Due to the activity of urease, adding urea is sufficient to prevent the transmembrane potential difference collapse observed below pH 3.5 (Meyer-Rosberg et al., 1996). Lactobacilli, however, are gram-positive lacking a periplasmic space, and have not been shown to release pH altering molecules, other than lactic acid itself.

Lactic acid is a potent microbicide

Although the production of lactic acid may slow the growth rate of vaginal Lactobacilli, it may also provide a selective advantage. Polymicrobial vaginal communities tend to fluctuate rapidly with one or more episodes of BV per month while the monomicrobial Lactobacilli communities tend to be stable over months to years (Brotman et al., 2010; Gajer et al., 2012). The stability of these communities may be due to the microbicidal actions of lactic acid at an acidic pH. Lactic acid is a potent and broadly active microbicide that provides significant protection to women with a vaginal bacterial community dominated by *Lactobacilli* spp. We and others have found that the vaginal concentration of lactic acid inactivates HIV (Aldunate et al., 2013), *Chlamydia trachomatis* (Gong et al., 2014) and HSV (Conti et al., 2009). In these studies the microbicidal activity of lactic acid was abolished at a neutral pH, however, an acidic pH without lactic acid was not as effective. Furthermore, the L-isomer of lactic acid was more potent for inactivating HIV while the D-isomer was more potent for inactivating *Chlamydia*

trachomatis. Taken together, these results suggest that the protonated form of lactic acid is microbicidal, but also that the structure of the molecule is important.

We have also shown *in vitro* that vaginal concentrations of lactic acid potently kill isolates of 17 species of BV-associated bacteria (O'Hanlon et al., 2011). Therefore, it may be lactic acid that prevents transitioning from a vaginal bacterial community dominated by *Lactobacilli* spp. to a polymicrobial community. Despite the strong microbicidal activity observed in this study *in vitro,* it is essential to show that effect can also be observed *ex vivo*. Bacteriocins represent an alternative hypothesis to lactic acid as to how Lactobacilli may prevent the growth and vaginal colonization of BV bacteria. Several bacteriocins are produced by specific *Lactobacilli* spp. that exhibit bacteriocidal effects against *G. vaginalis*, a key BV-associated bacterial species (Stoyancheva et al., 2014). However, these tests were only performed at a neutral pH and bacteriocins may be unnecessary for the inactivation of BV bacteria in the presence of a high concentration of lactic acid.

Not only are vaginal bacterial communities dominated by *Lactobacilli* spp. near monomicrobial, but the each tends to consist of a single species and possibly strain of vaginal Lactobacilli. One explanation for this phenomenon may be that Lactobacilli themselves can be inactivated if the acidity drops below a certain point. If numerous strains of Lactobacilli were present, only the most acid tolerant would survive.

Research Goals

I: Do vaginal Lactobacilli continue to grow and acidify under the acidic conditions of the vagina?

To understand how vaginal Lactobacilli continue to dominate the vaginal bacterial community, and maintain a stable vaginal pH, I wanted to determine if vaginal Lactobacilli could be observed growing and acidifying *ex vivo* at the *in vivo* vaginal pH. Samples obtained *ex vivo* by inserting and removing a softcup contain mostly luminal bacteria, as well as some bacteria bound to the outer layer of the vaginal epithelial cells that were scraped off. Observing whether this population of bacteria was growing was important to determine if vaginal Lactobacilli could tolerate and continue to grow directly in its acidic environment, unlike *H. pylori*, or if the growing population was sequestered deeper in the vaginal epithelium.

II: Does overacidification of CVF lead to the inactivation of Lactobacilli?

At high vaginal concentrations of lactic acid the bacterial community is near monomicrobial, usually comprising a single *Lactobacilli* spp. This likely occurs as a strain of Lactobacilli achieve stable vaginal acidity that inactivates all other bacteria until this dominant strain is all that remains. I wanted to understand what happens to these Lactobacilli if the vaginal acidity increases beyond what they were exposed to *in vivo*. Knowing if vaginal Lactobacilli are inactivated by overacidification is important for understanding both the mechanism by which vaginal Lactobacilli reach a stable asymptotic vaginal pH, as well as why vaginal Lactobacilli usually exist in a near monomicrobial community.

III: Is vaginal lactic acid sufficient to inactivate BV bacteria *ex vivo*?

Vaginal lactic acid is a potent microbicide and inactivates BV-associated bacteria *in vitro*. H_2O_2, however, was also shown to be a potent microbicide *in vitro*, but was not efficacious *ex vivo*. By obtaining BV bacteria *ex vivo* I wanted to verify the physiological significance of the microbicidal activity of lactic acid. Furthermore, although bacteriocins produced by vaginal Lactobacilli have been discovered, I believed that the acidity of the CVF due to lactic acid was sufficient to inactivate BV bacteria. By dialyzing CVF obtained from women with low Nugent scores against saline, I removed lactic acid from CVF. Using the original CVF and the dialyzed CVF allowed me to determine whether or not lactic acid is sufficient for the inactivation of BV bacteria.

Materials & Methods

CVF Collection

All CVF samples were self-collected by donors by inserting and removing an Instead® SoftCup menstrual collection device (Boskey et al., 2003). Softcups were placed in 50 mL conical tubes and centrifuged to collect the CVF. All samples were gram stained and observed under the microscope to assign a Nugent score (Nugent et al., 1991). In the majority of experiments only samples with Nugent score of 0-3 were used to ensure that the results reflected the behavior of vaginal Lactobacilli, and not a polymicrobial vaginal community. For the BV bacteria inactivation experiments only samples with a Nugent score of 7 or higher were used.

CVF video microscopy and bacterial growth rate determination

CVF was lightly applied to 10 uL 1.5% agarose pads which were then sealed in a slide chamber under anaerobic conditions. In some experiments, NaOH was added to alkalinize the pH of CVF to pH 6. Typically, the volume of NaOH was close to but no more than 1 tenth of the volume of the sample. Live bacteria were visualized on a Zeiss Axiovert 200 inverted light microscope using a DIC filter at 63x magnification within a temperature controlled chamber.

The lengths of at least 30 bacterial chains were measured over a four hour period using ImageJ. By normalizing elongation rates to the original length of the chain, percent elongation rates were obtained, and converted to doubling times. A single average doubling time was calculated for each bacterial chain.

For planktonic growth experiments CVF samples were rinsed twice in a 10-fold volume of saline before being resuspended in MS growth medium of varying pH. Amphotericin B was added to the cultures to prevent fungal growth. Cultures were placed in a sealed 96-well plate and the OD600 was monitored over time at 37°C. Given that the pH of these cultures would not remain constant over time, only the initial rate of change in OD600 (between 0 and 6 hours) was measured.

Measuring the lactic acid concentration and pH in CVF samples incubated over time

Freshly obtained unmodified CVF was quickly exposed to a hypoxic environment in glove-box transferred to a single test tube, before being sealed and placed in a 37°C incubator. Over time the tube was removed from the incubator and its pH was determined using a microelectrode. To maintain the hypoxic environment, pH measurements were only performed within the glove-box. Aliquots of CVF were taken at each timepoint and frozen. Frozen samples were thawed and spun to obtain CVF supernatants from the various timepoints. The lactic acid concentration of these supernatants was measured using the D-lactic acid/L-lactic acid Enzymatic BioAnalysis/Food Analysis UV method kit (R-Biopharm, Darmstadt, Germany), and a plate spectrometer. Typically samples were diluted 1:30 for the signal to be in the linear range of the spectrometer.

Bacterial inactivation assays

Saline and lactic acid mixtures were added to fresh CVF in a 1:5 ratio before incubation at 37°C under hypoxic conditions. In samples where the pH was kept constant, the saline and lactic acid mixtures were titrated beforehand to match the pH of the sample. After two hours of

incubation the samples were diluted 1:10 in a 50 mM HEPES saline solution at pH 7. Samples were serially diluted w/v in saline then plated on Brucella broth, 5% sheep blood plates. Blood plates were chosen to ensure that most vaginal species of bacteria would be able to grow. Blood plates were incubated at 37°C in anaerobic jars with oxygen scavenger sachets. After 2-3 days of incubation, CFUs were counted.

CVF microdialysis

CVF samples from women with low Nugent scores were somewhat diluted (1:10) to thin the mucus, and supernatants obtained by centrifugation were then sterile filtered using 0.2 micron spin filters. Sterile CVF was pipetted into dialysis chambers and sealed with a square of 2 kDa MWCO dialysis membrane. Chambers were floated on dialysate that was gently stirred for 2 hours.

Results

Vaginal Lactobacilli species tolerate and continue growing at the vaginal pH

Non-vaginal species of Lactobacilli including *L. delbrueki, L. acidophilus,* and *L. paracasei* optimally grow in culture at neutral or near-neutral pH (Adamberg, 2003). If vaginal Lactobacilli achieve a steady vaginal pH, but their growth is determined by the pH, vaginal Lactobacilli must continue to grow and acidify even while exposed to the acidity of CVF. To test this I directly observed *ex vivo* vaginal Lactobacilli growing in CVF. Using video microscopy I measured the doubling time of bacteria in unmodified CVF obtained from individuals with a Nugent score of 0-3 (**Fig 1A, images: Fig 2**). Across all the samples it was estimated that at least 95% of the bacteria were elongating at a rate that doubled their length in about 12 hours. If the growth rate of vaginal bacteria is determined by the vaginal pH I would expect the doubling of *ex vivo* Lactobacilli in CVF to increase if the pH of the CVF was artificially raised. As expected raising the pH of CVF to 6 through the addition of NaOH shortened the doubling time of the bacteria. On average the doubling time was about 3-fold shorter in alkalinized CVF compared to unmodified CVF. This trend was also true if the CVF samples were cultured in media. I measured the growth rate of *ex vivo* vaginal bacteria in MS, a Lactobacilli-restrictive growth medium, at an initial pH between 4 and 6, using OD600. In each cultured sample the highest growth rate was observed in cultures with an initial pH of 6, and the lowest growth rate was observed in cultures with an initial pH of 4 (**Fig** 1B).

Vaginal bacteria continue to acidify *ex vivo*

Lactic acid fermentation is necessary for Lactobacilli to produce energy. If the vaginal bacteria are still growing in the acidic environment of the vagina, they must continue to produce lactic acid. As expected, anaerobic incubation of CVF samples from women with Nugent scores 0-3, caused the pH to decrease over time, as the lactic acid concentration increased (**Fig 3**). On average the bacteria continued to produce lactic acid at a rate of 13 mM/hr. Not surprisingly, an increase in the lactic acid concentration caused a decrease in the pH of the CVF, which can be observed over time. The pH *ex vivo* continues to drop, despite our previous finding that the *in vivo* CVF pH is stable. This suggests that the lactic acid being produced *in vivo* is lost from the vagina, likely by crossing the vaginal epithelium.

Concentrations of lactic that exceed the vaginal pH can limit the growth or inactivate vaginal Lactobacilli

Matching the rate of lactic acid production to the loss across the epithelium provides an explanation as to how a stable pH is achieved, however, it remains unclear why some strains reach a lower pH than others. At least two possibilities exist: 1) Host factors such as the shedding rate of the vaginal epithelium, or the permeability of the vaginal epithelium to lactic acid determines the stable vaginal pH; 2) Vaginal strains of Lactobacilli acidify as far as they are able without overly slowing their own growth rate or inactivating themselves. The second possibility is supported by our previous experiments. When we incubated CVF samples both the production of lactic acid, and the change in pH plateaued between 5 and 10 hours, suggesting eventual inactivation of the bacteria. Given that the vaginal Lactobacilli grow more slowly at an acidic pH, I wanted to test if additional lactic acid eventually caused inactivation. I increased the

lactic acid concentration of fresh CVF and measured the fraction of CFUs remaining after two hours of incubation at 37°C. In each sample, any additional lactic acid reduced the number of CFUs compared to incubation with saline (**Fig 5**). The high concentrations of lactic acid reduced the concentration of CFUs to less than were observed before incubation, suggesting that at these levels the bacteria were inactivated. I also increased the lactic acid concentration without lowering the pH by using lactic acid that had been titrated to match the pH of the sample. Similar inactivation was observed, suggesting that the concentration of weak acid, and not just the pH is important.

Vaginal lactic acid is sufficient to kill BV-associated bacteria

Lactic acid is a potent microbicide, and we and others have shown that vaginal levels can inactivate HIV (Aldunate et al., 2013), *C. trachomatis* (Gong et al., 2014) and HSV (Conti et al., 2009) as well as BV-associated bacteria *in vitro* (O'Hanlon et al., 2011). During the development of HIV microbicides for vaginal protection, many candidates that potently inactivated HIV in vitro, were strongly inactivated by CVF and/or semen, most notably in the case of H_2O_2. Therefore, I sought to determine whether lactic acid was effective against BV-associated bacteria in the presence of CVF. Recent discoveries suggest that Lactobacilli produced bacteriocins play a role in fending off other bacteria (Stoyancheva et al., 2014), however, I believe that lactic acid is sufficient at the vaginal pH to inactivate BV bacteria. To test this I sterile-filtered CVF from women with Nugent scores 0-3 and performed dialysis across a 2 kDa MWCO membrane. Dialysis against saline was successful in removing nearly 95% of the lactic acid. Incubation of *ex vivo* BV bacteria with the undialyzed CVF reduced the concentration

of CFUs by 100-fold on average, whereas no significant reduction in CFUs was observed if the CVF was dialyzed against saline (**Fig 6**). Many small molecules including lactic acid will be lost across a 2 kDa MWCO membrane. Therefore, to show that lactic acid alone, is sufficient to inactivate BV bacteria, I also dialyzed the CVF from low Nugent women against a saline solution with a matched lactic acid concentration to the original sample. The CVF that retained its lactic acid caused levels of inactivation that were not significantly different than the undialyzed CVF. This confirms that vaginal lactic acid is sufficient to inactivate BV-associated bacteria in CVF. To determine if the potency of lactic acid was only due to pH I also dialyzed against a saline solution that lacked lactic acid, but was matched to the pH of the original sample. The CVF that was depleted of lactic acid, but retained its acidic pH did not inactivate BV bacteria.

Discussion

I have determined that the growth rate of vaginal Lactobacilli is dependent on the vaginal pH. This is in agreement with the ATCC strain of *L. gasseri* and other *Lactobacilli* spp. (Adamberg, 2003; Boskey et al., 1999). Furthermore, at the stable vaginal pH the Lactobacilli are still growing, albeit 3 times slower than if the vaginal pH was 6. This is in marked contrast to *H. pylori* which has adapted to avoid or counter the acidity of the stomach. Instead, Lactobacilli survive and grow while directly exposed to the acidity of the environment, which they produce.

A possible reason for why Lactobacilli slow their growth rate is to divert more energy toward an acid-resisting response, however, our data suggests it also prevents vaginal Lactobacilli from creating an environment that is too harsh to survive. In each of our CVF samples an increase in the lactic acid concentration inhibited growth or caused bacterial inactivation regardless of the starting concentration or pH of the CVF. Too many Lactobacilli could acidify too quickly for the population to survive. This strongly suggests that the vaginal asymptotic pH is selected for as the lowest pH the bacteria can survive. Overall, it is likely beneficial for the vaginal Lactobacilli to create an environment that is just barely tolerable, but contains a high concentration of lactic acid.

Here I report that lactic acid alone is sufficient to inactivate *ex vivo* BV bacteria in CVF. In agreement with the *in vitro* study from our lab (O'Hanlon et al., 2011), lactic acid at an acidic pH caused more inactivation of BV bacteria than an acidic pH alone. The *in vitro* study and *ex vivo* experiments presented here differ in the degree of bacterial inactivation. O'Hanlon reported inactivation between 4 and 5 log units, while only a 2 log unit inactivation was observed *ex vivo*. This could reflect differences between the *ex vivo* BV bacteria and the ATCC strains of BV-

associated bacteria. Actual BV bacteria are more likely to have been challenged by Lactobacilli or lactic acid in the past, and may be more resistant. Furthermore, as all of these experiments took place in CVF, it is also possible that the high concentration of energy sources allowed BV bacteria to rapidly metabolize and defend themselves against lactic acid.

Although lactic acid is sufficient to inactivate BV bacteria, this data does not rule out a role for vaginal Lactobacilli bacteriocins, but suggests they would be unnecessary at an acidic pH. Instead, they may be important during times of alkalization such as ejaculation or menses to fend off other bacteria. As most bacteriocins are species specific, it would make sense for Lactobacilli bacteriocins to play a specific role in the vagina, while lactic acid remains a broad-spectrum microbicide.

These results in total provide a model by which a steady-state vaginal pH is created and maintained by vaginal Lactobacilli. In response to neutralization, by either ejaculation or menses, the growth rate of vaginal bacteria would quickly increase, as was observed *ex vivo* with NaOH. Increasing the growth rate would likely increase the number of vaginal Lactobacilli and therefore the rate of acidification. As the vagina reacidifies the growth rate would begin to slow. If the rate of acidification remained too high, the pH would go below what the bacteria could tolerate and the growth rate would get too low to support the population. Less bacteria would lower the acidification rate until it matched the loss of acid across the vaginal epithelium, creating a steady-state population.

This model is useful to understand a key observation of vaginal Lactobacilli. In most women only one species of Lactobacilli is present in high numbers. This would be expected given our model. If two species of Lactobacilli were present at first, the species that acidified to

and could survive the lower pH would eventually outnumber the other. In terms of Lactobacilli probiotic design, we would expect a vaginal strain with a low asymptotic pH to be more beneficial in preventing BV, and potentially more stable in the long-term due to its superior acid tolerance.

An implication of this model is that the lowered growth rate of the bacteria at the asymptotic vaginal pH must also match the loss-rate of bacteria due to vaginal epithelium shedding. Some groups have claimed that the rate of vaginal epithelial shedding is faster in women with BV (Gilbert et al., 2013), however, data presented in the next chapter may contradict this. Interestingly, lubricant use is associated with episodes of BV in individuals with less stable vaginal populations. Most lubricants are hypertonic and cause excessive shedding of the vaginal epithelium (in preparation). This would be detrimental to a low pH, slow growing Lactobacilli population. A faster shedding rate could only accommodate a Lactobacilli population growing at a higher pH, and therefore growing faster.

In the next chapter I present data that a vaginal community dominated by *L. crispatus* correlates with a lower pH than a vaginal community dominated by *L. iners*. Interestingly, *L. iners* samples have a higher concentration of bacteria than *L. crispatus* samples (unpublished observation), adding support to a connection between bacterial growth rates and the vaginal pH, and predicting that shedding rates are likely different as well. What remains unclear is if this is due to variable shedding rates between individuals, or if the shedding rates could be linked to the pH. One study has shown that increasing the pH of skin above 5 increases the shedding rate (Lambers et al., 2006), offering the possibility that Lactobacilli may lower the shedding rate through the production of lactic acid. Understanding the variability of epithelium

shedding will be very important in determining which probiotic approach and strains will be best.

Chapter figures

Figure 1—The growth rate of vaginal Lactobacilli decreases as the pH decreases
Doubling times of vaginal Lactobacilli in unmodified *ex vivo* CVF and CVF alkalinized to pH 6 as determined by video microscopy (A); Growth rate in titrated growth media as determined by the change in light scattering (OD 600) relative to the growth rate at pH 6 (B)

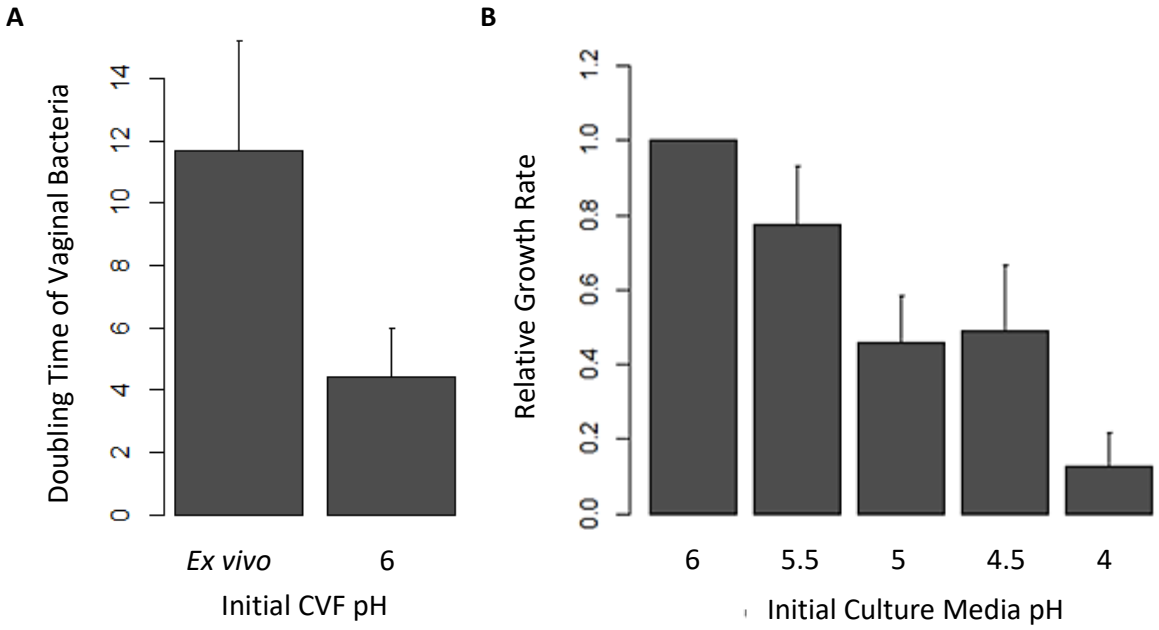


Figure 2—Video microscopy demonstrating the growth of vaginal Lactobacilli *ex vivo*
Video microscopy of vaginal Lactobacilli incubated in unmodified *ex vivo* CVF (top) or CVF alkalinized with NaOH to pH 6 (bottom) demonstrating slow bacterial elongation at near vaginal pH and much more rapid elongation at pH 6. The arrows point to the position of one end of selected bacteria at t=0.

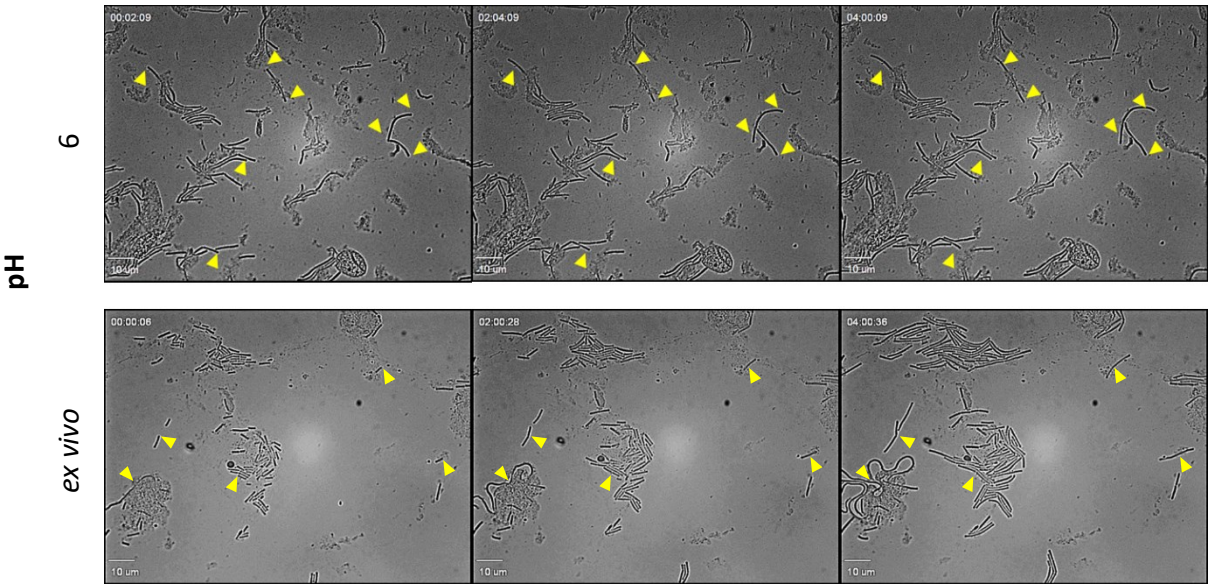


Figure 3—Incubated *ex vivo* vaginal Lactobacilli continue to produce lactic
Concentration of lactic acid in unmodified *ex vivo* CVF obtained from donors with Nugent = 0-3 incubated hypoxically at 37°C over time. Each line represents a unique donor. Dotted lines corresponds to the average linear increase in lactic acid concentration over the first 5 hours.

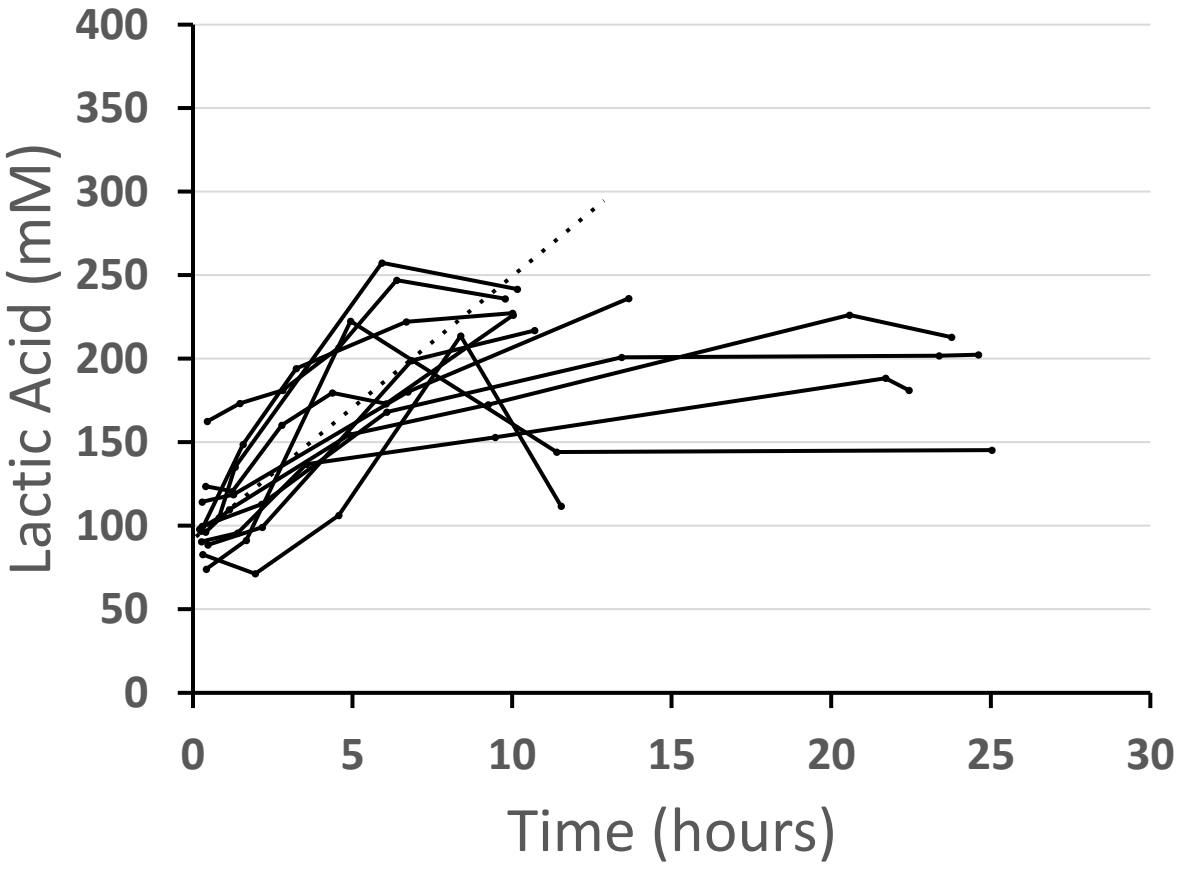


Figure 4—The pH of unmodified *ex vivo* CVF decreases when incubated

CVF obtained from donors with Nugent = 0-3 incubated hypoxically at 37°C over time. Each line represents a unique donor.

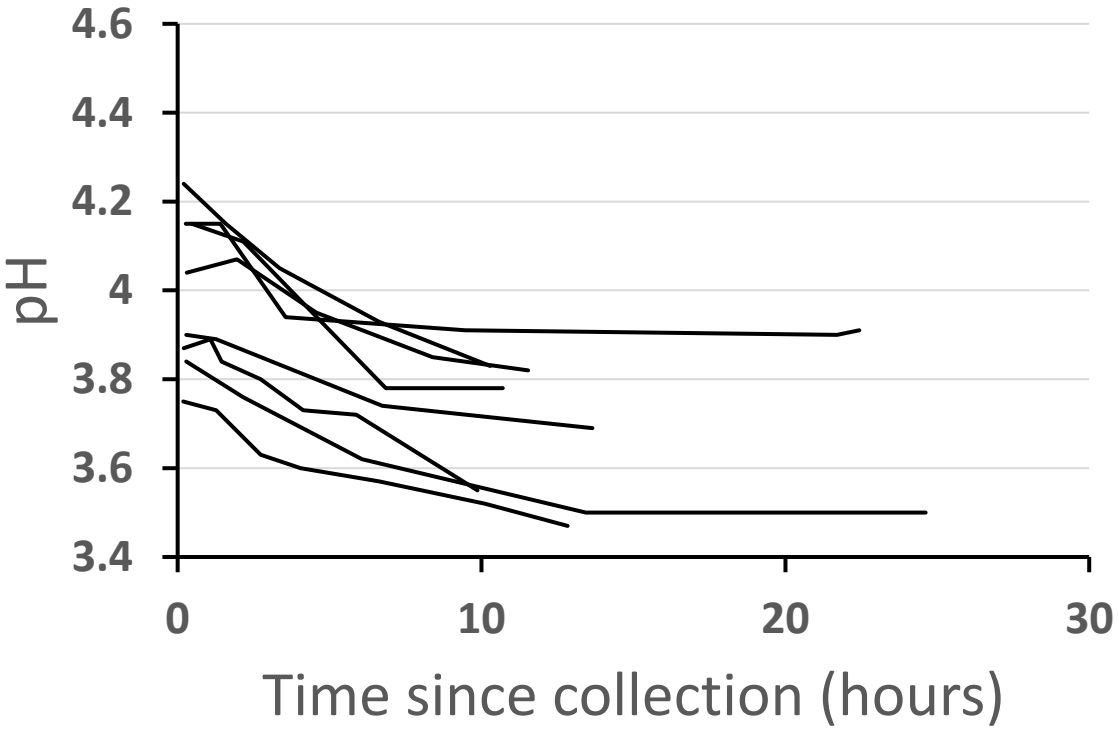


Figure 5—Additional lactic acid beyond the *in vivo* concentration inactivates vaginal Lactobacilli

Log of the fraction of CFUs remaining after the addition of lactic acid to fresh CVF and 2 hours of incubation at 37°C, as compared to adding only saline. t=0 refers to the initial fraction of CFUs compared to the saline incubation, before the addition of lactic acid or incubation.

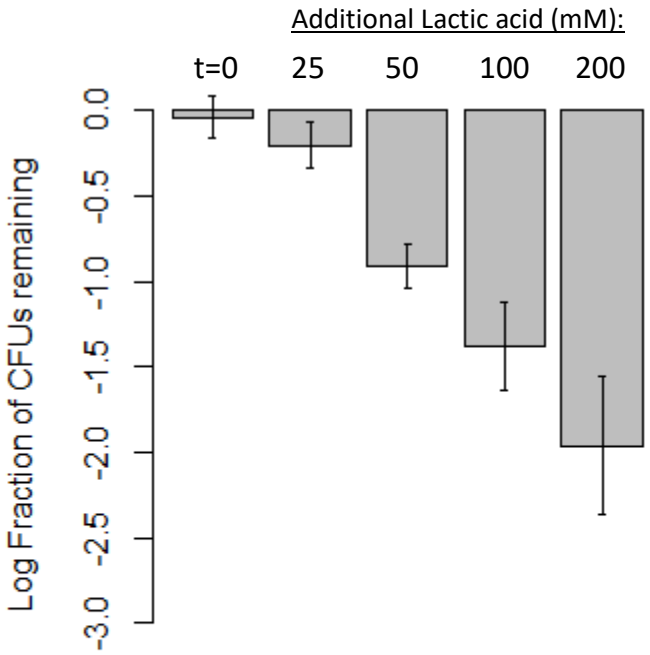
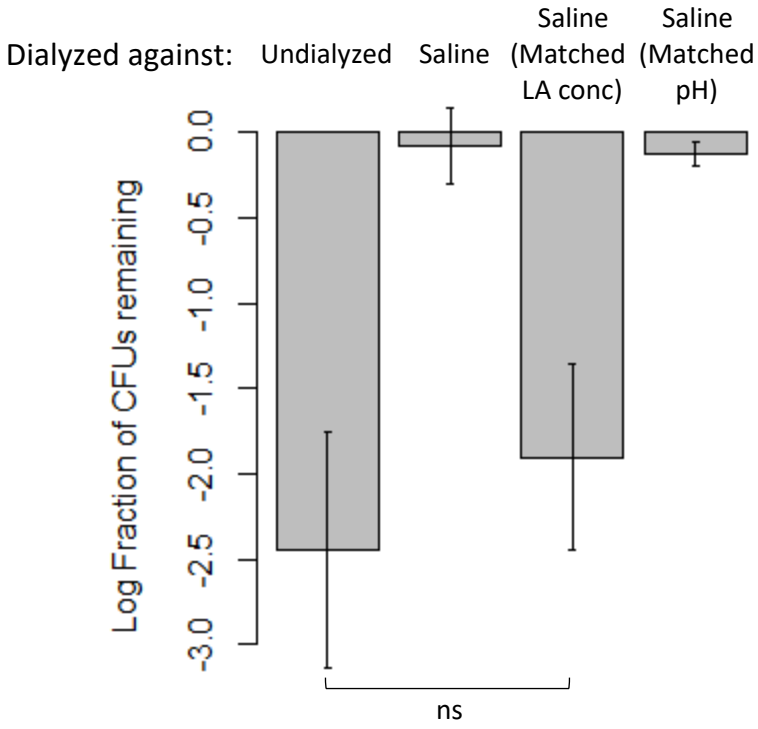


Figure 6—Lactic acid is sufficient to inactivate BV bacteria *ex vivo*

Log of the fraction of CFUs after incubation of *ex vivo* BV bacteria with dialyzed CVF from low Nugent individuals compared to saline. CVF was either left undialyzed or was dialyzed against saline, saline with the same pH and lactic acid concentration as the undialyzed CVF and saline with the same pH as the undialyzed CVF.



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Vaginal acidity and physiology as a function of specific bacterial communities

Chapter abstract

The vaginal microbiota falls into one of two major groups: near monomicrobial *Lactobacillus* species, or polymicrobial communities. Epidemiological studies have shown that women who lack a predominant *Lactobacilli* species are at a higher risk for STI transmission. Interestingly, even amongst women with a predominant *Lactobacilli* species, there's a higher risk associated with *Lactobacilli* spp. that do not produce H_2O_2, compared to those that do. Community sequencing has revealed that amongst women with a predominant *Lactobacilli* spp., the vaginal microbiota comprises three community state types (CSTs), each defined by a specific species of *Lactobacilli* spp. Polymicrobial communities, which manifest clinically as Bacterial Vaginosis (BV) comprise two CSTs, each of which lack the predominance of *Lactobacilli* spp. We have previously established that H_2O_2 is not the microbicidal agent in CVF. Instead, lactic acid and a low pH allow for the inactivation of HIV and *C. trachomatis*. Therefore, in these experiments I attempted to determine if the concentration of lactic acid and the pH of CVF could explain previous epidemiologic results. I found that the CVF of women with polymicrobial CSTs lacks significant lactic acid, and has too high a pH to be protective despite the presence of *Lactobacilli* spp. within these samples. The lowest pHs were observed in samples identified as CST I-CRISP, containing *L. crispatus* in agreement with H_2O_2 producing *Lactobacilli* spp. having the least risk. On the other hand, CST III-INERS samples, containing *L. iners* that does not produce H_2O_2 had a

broad pH range that overlapped with the pH range of both CST I-CRISP samples and polymicrobial samples. Strong correlations were observed between the pH and lactic acid concentration across all CSTs. The broad ranges of CST III-INERS, which extend into the ranges found for the polymicrobial samples confirm that there is a strong correlation between the epidemiologic studies and the acidity of CVF across different CSTs. Furthermore, these data provide insight as to why some CSTs may be more or less likely to switch to others.

Background Information

Vaginal Bacteria Community State Types

Advances in high-throughput sequencing allowed the entire vaginal microbiome to be characterized through sequencing of amplified 16S rDNA regions obtained from an entire community of vaginal bacteria. With this approach, the classifications of predominant *Lactobacilli* spp. and a polymicrobial community were both more clearly defined and further characterized by the variability within each. Three independent studies found that the vaginal microbiome is either polymicrobial, or represented by a single predominant *Lactobacilli* spp. that was either *L. crispatus, L. iners, L. jensenii or L. gasseri* (Hummelen et al., 2010; Ravel et al., 2011; Spear et al., 2011). The nomenclature and breakdown of Community System Types (CSTs) provided by Ravel et al. (2011) has largely been adopted by the field. Specifically, vaginal samples were identified as one of 5 bacterial CSTs. CSTs I, II, III and V were represented by a predominant *Lactobacilli* spp. and CST IV was characterized by both the absence of a predominant *Lactobacilli* spp. and a polymicrobial array of gram-negative species of bacteria, including *G. vaginalis* which has long been associated with bacterial vaginosis (BV). In this document these names will be used to describe different vaginal CSTs, however, for simplicity the abbreviated name of the predominant bacteria will be included: CST I-CRISP, CST II-JENS, CST III-INERS, CST IV-POLY and CST V-GASS.

Each of the independent 16S sequencing studies revealed that the four CSTs with a predominant *Lactobacilli* spp. are near monomicrobial (Hummelen et al., 2010; Ravel et al., 2011; Spear et al., 2011). This was especially true for CST I-CRISP, CST II-JENS and CST V-GASS. In

these CSTs about 95% of bacterial sequence reads belonged to one species of *Lactobacilli*. CST III-INERS was similar, but had more additional species.

Nugent scoring and vaginal bacterial community prevalence

As discussed in the introduction, estimates for the prevalence of BV, and by proxy the polymicrobial CSTs, relied on Nugent scoring. Nugent scoring attempts to distinguish BV by a lack of Lactobacilli morphotypes and the presence of BV-associated bacteria morphotypes (Nugent et al., 1991). It is calculated as the sum of scores associated with the presence or absence of these bacteria inferred by morphology. The score includes the amount of Lactobacilli or gram-positive rods, and the amount of BV-associated bacteria which are gram-negative. The presence or absence of *Mobiluncus* spp. also contributes to the score, although the utility of this is less clear today. Using Nugent scoring the largest BV prevalence study to date found that 29% of women in the United States had BV, as diagnosed by a Nugent score of 7-10 (Kenyon et al., 2013; Koumans et al., 2007). An additional 33% of women were labeled "intermediate" with a Nugent score of 4-6, and the remaining 38% were labeled "healthy" with a Nugent score less than 4.

A Nugent score of 4-10 would require the presence of more than just Lactobacilli morphotypes, but Lactobacilli dominated CSTs are usually near monomicrobial. Therefore, under our current understanding of vaginal bacteria CSTs, we would expect women with a Nugent score of 4-10, or 62% of US women (Koumans et al., 2007) to be polymicrobial. A potential exception to this may be *L. iners*. Unlike the other vaginal *Lactobacilli* spp. *L. iners* despite being gram-positive, is frequently gram-negative in appearance (unpublished

observations). Therefore, some number of samples scored as intermediate or BV by Nugent score, may actually contain high concentrations of *L. iners* that are morphologically more similar to BV-associated bacteria and misidentified. Women with a Nugent score of 0-3, or 29% of US women, likely have a *Lactobacilli*-dominated CST, as a score that low requires a predominance of Lactobacilli morphotypes and little else.

Community State Type stability and transitioning

CSTs are relatively stable, although a transition is most likely to occur before ovulation or before menses when estrogen levels are low (Gajer et al., 2012). These transitions have been well documented (Brotman et al., 2010), and certain CSTs transition to others at different rates (Gajer et al., 2012). Broadly speaking, Lactobacilli dominated CSTs are more stable than the polymicrobial community. CST III-INERS is the most likely CST to transition to CST IV-POLY. CST III-INERS also transitions to CST I-CRISP, but at a lower rate. CST I-CRISP, however, is not very likely to transition to CST IV-POLY, and transitions to CST III-INERS more frequently than any other CST. Taken together, this strongly suggests that the bacteria present in the different CSTs create a vaginal environment that is more hospitable to a specific subset of bacteria from other CSTs.

Risk Factors Associated with Vaginal Bacterial Communities

Although Nugent scoring only identified about one-third of women as having BV, it is important to note that most epidemiological studies have shown that the risk of STI infection associated with BV extends beyond women with a Nugent score of 7-10 and includes women who are “intermediate” with a Nugent score of 4-6 (Cohen et al., 1995; Martin et al., 1999).

Therefore the risk association is not limited to BV but to any vaginal bacterial community where *Lactobacilli* spp. are not predominant, as determined by Nugent scoring. Despite Nugent scoring only identifying 29% of the United States as having BV, the risk of STI acquisition would apply to the remaining 62% of women.

Higher acidity is likely to be superior at inactivating STI pathogens

It is likely that the risk of STI infection associated with a polymicrobial vaginal community is due to the lack of lactic acid compared to women with a Lactobacilli dominated vaginal community. This agrees with the dose-dependent microbicidal activity of lactic acid against HIV (Aldunate et al., 2013), *C. trachomatis* (Gong et al., 2014) and HSV (Conti et al., 2009), as well as with the association of risk with any vaginal bacterial community where *Lactobacilli* spp. are not predominant.

Physiological differences in CVF between vaginal CSTs

Surprisingly, few studies that incorporate 16S sequencing have attempted to identify correlative physiological differences in the CVF of women with different vaginal CSTs, opting instead for associations with health risks. Two studies that did compare the CVF between women with different CSTs assayed the vaginal glycogen content in CVF (Mirmonsef et al., 2014) and the ability of the CVF to trap HIV particles (Nunn et al., 2015). Glycogen levels were found to be highest in samples that were identified as CST I-CRISP or CST III-INERS as opposed to CST IV-POLY, and CST I-CRISP had a higher concentration than CST III-INERS. HIV trapping was achieved in CST I-CRISP, but not CST III-INERS or CST IV-POLY.

Most surprisingly, even differences in the pH and lactic acid concentration between different vaginal CSTs have not been thoroughly investigated. Ravel et al. attempted to compare the average pH of the CVF in the different CSTs, however, their pH assay did not allow for measurements below 4.0 (2011). Even with this limitation, they observed that CST III-INERS had a higher average pH than CST I-CRISP. Mirmonsef et al. (2014) and Nunn et al. (2015) both supported and disagreed, respectively, with the finding that CST III-INERS had a higher average pH than CST I-CRISP. Only Nunn et al. (2015) measured the lactic acid content, and found it was not significantly different between CST I-CRISP and CST III-INERS.

Research Goals

I: Within Lactobacilli dominated communities are some *Lactobacilli* spp. more likely to offer protection than others? Does STI acquisition risk correlate with vaginal acidity?

If the risk of STI infection is due to a lack of lactic acid, and women who lack H_2O_2 producing bacteria are at a higher risk, I expected women in CST I-CRISP or CST II-JENS, characterized by the predominance of H_2O_2 producing *L. crisptatus* or *L. jensenii,* respectively, to have a lower pH and higher lactic acid concentration than CST III-INERS or CST V-GASS, which are characterized by *L. iners* and *L. gasseri* which do not produce H_2O_2.

II: Do lactic acid concentrations and pH values of specific communities offer insight into how community transitions occur?

As discussed previously in this dissertation, lactic acid is sufficient to inactivate polymicrobial vaginal communities. Therefore, if different CVF lactic acid concentrations are observed between different CSTs, it may provide evidence for why certain CSTs are more likely to switch to others. Specifically, given that CST III-INERS is most likely to switch to a polymicrobial CST, I expected that the lactic acid concentration of CST III-INERS CVF samples would be lower than the other *Lactobacilli*-dominated CSTs.

III: Beside pH and the concentration of lactic acid, are there physiological differences between CVF from women with different vaginal CSTs?

Few studies have approached variation in CVF with an eye toward bacterial CSTs, despite the variability of risk observed between different CSTs. Therefore, in association with

measuring the acidity of CVF samples that were to be included in a 16S sequencing dataset, we also measured the osmolality, cell count and protein concentration.

Materials & Methods

Sample collection and processing

Voluntary donors were recruited at the Baltimore Druid Hill Health Clinic by cooperating medical staff. All CVF samples were self-collected by donors by inserting and removing an Instead® SoftCup menstrual collection device (Boskey et al., 2003). Softcups were placed in 50 mL conical tubes and centrifuged to collect the CVF. Vaginal swabs were also self-administered and stored at -20°C before being used for 16S RNA community sequencing. The weight of each CVF sample was determined. Depending on the size of the sample it was allocated into separate tubes for the lactic acid concentration, protein concentration and osmolality measurements, and stored at -80°C. All samples were gram stained and observed under the microscope to assign a Nugent score (Nugent et al., 1991).

16S RNA community sequencing

16S sequencing was performed by the Institute for Genome Sciences (IGS), University of Maryland in accordance with their previously published methods (Gajer et al., 2012). Briefly, genomic DNA extracted from frozen vaginal swabs was used for PCR amplification of the V1-V2 region of the 16S RNA gene. Purified amplicons were sequenced by 454 pyrosequencing, and quality control of sequence reads was performed with QIIME (Caporaso et al., 2010). The Taxonomic assignments were made with a combination of the Ribosomal Database Project (RDP) Classifier (Wang et al., 2007) and speciateIT. We thank Jacques Ravel for extending the services of the IGS to our study.

Data analysis and CST Assignments

Samples were excluded from analysis if the total number of reads was less than 600. The dataset was then rarefied in R, using the lowest number of reads for any given sample, 1061, and read counts were converted to the log of the fraction of the total number of reads per sample.

CST assignments were made in R, using the methods described in (DiGiulio et al., 2015). Breifly, principal component analysis and the gap statistic function from the R stats cluster package were used to estimate the number of CSTs (Maechler et al., 2016). The analysis revealed 4 clusters, and clustering was performed using the partitioning around medioids algorithm (Maechler et al., 2016). The four resulting CSTs were named in accordance with Ravel et al. (2011), including grouping both polymicrobial CSTs into the singular CST IV-POLY. The R stats platform ggplot2 (Wickham, 2009) was used to generate the heatmap of the data.

Measuring the pH, the lactic concentration, the protein concentration and the osmolality in CVF

Measurements of pH were made with a microelectrode, in whole CVF before freezing. Measurements were not made under hypoxic conditions, or in the presence of 5% CO_2. The lactic acid concentration of CVF supernatants was measured using the D-lactic acid/L-lactic acid Enzymatic BioAnalysis/Food Analysis UV method kit (R-Biopharm, Darmstadt, Germany) and a plate spectrometer. Typically samples were diluted 1:30 for the signal to be in the linear range of the spectrometer. The protein concentration of CVF supernatants was measured using the Thermo Scientific Pierce BCA Protein Assay Kit and a plate spectrometer. Typically samples

were diluted 1:50 for the signal to be in the linear range of the spectrometer. Osmolality measurements were made using undiluted CVF supernatant and a VAPRO® 5520 vapor pressure osmometer (ELITech Group, Logan, UT).

Results

Bacteria Community Assignments

35 vaginal swabs and CVF samples were obtained from patients of the Baltimore City Druid Hill Clinic. The vaginal swabs were used for 16S RNA sequencing and cluster analysis was used to assign CSTs to each sample (**Fig 7**). The samples were clustered according to the fraction of reads that could be assigned to a specific bacterial species. Unlike Ravel 2011, CST II-JENS and CST V-GASS were not observed in our sample set. As these CSTs are the least represented in their dataset we expect this is due to the lower number of samples.

As can be seen, CST I-CRISP was heavily predominated by *L. crispatus* and very few other species. CST III-INERS was similar with the predominant *L. iners*, however, the BV-associated *G. vaginalis* was more highly represented in each of these samples compared to CST I-CRISP. Although, CST IV-POLY lacked a predominant *Lactobacilli* spp., *L. iners* was still detected in the majority of these samples, while other *Lactobacilli* spp. were not.

Vaginal acidity greatly varies between different CSTs

The epidemiological data claims that both polymicrobial communities and Lactobacilli dominated communities that lack H_2O_2 producing Lactobacilli, are at a higher risk for STI acquisition. It is unlikely that this is due to the production of H_2O_2 (O'Hanlon et al., 2011). Instead, given the broad microbicidal activity of lactic acid, differences in vaginal acidity likely correlate with elevated risk for STI acquisition. Using the CST assignments, vaginal acidity comparisons were made between the different vaginal communities (**Fig 8**). Not surprisingly,

the lowest vaginal acidity was observed in CST IV-POLY. The pH of CST IV-POLY samples ranged 4.61-5.51 with a mean pH of 5.06 ± 0.27. The lactic acid concentration in CST IV-POLY samples ranged 1.6-6.8 g/L with a mean concentration of 3.8 ± 1.4 g/L. The highest vaginal acidity was observed in CST I-CRISP. The pH of CST I-CRISP samples ranged 3.75-4.24 with a mean pH of 3.95 ± 0.19. The lactic acid concentration in CST I-CRISP samples ranged 8.8-14.1 g/L with a mean concentration of 10.5 ± 2.3 g/L. Interestingly, an intermediate vaginal acidity was observed in CST III-INERS. The pH of CST III-INERS samples ranged 4.09-5.46 with a mean pH of 4.68 ± 0.40. The lactic acid concentration in CST III-INERS samples ranged 1.9-9.8 g/L with a mean concentration of 5.4 ± 3.1 g/L. The wide range of acidity observed in CST III-INERS samples overlapped with both CST I-CRISP and CST IV-POLY. Two of the CVF samples assigned to CST III-INERS did not have a plurality of *L. iners*, but *G. vaginalis* instead (**Fig 7**). Importantly, these two samples are not the maxima of either the pH or lactic acid concentration range. Plotting the pH as a function of the lactic acid concentration in all CVF samples reveals a clear correlation (**Fig 9**), while visually revealing the wide range of vaginal acidity observed in CST III-INERS samples that extends from CST I-CRISP samples to CST IV-POLY samples.

Nugent scoring discriminates between Lactobacilli that produce high concentrations of lactic acid and bacteria that produce low concentrations of lactic acid

Nugent scoring was highly discriminant of bacterial communities CST I-CRISP and CST IV-POLY, but not CST III-INERS (**Fig 10**). All CST I-CRISP samples were considered healthy by Nugent score (0-3), while all CST IV-POLY samples were considered intermediate or BV-positive (4-10). Three of the CST III-INERS samples were assigned healthy Nugent scores, while the other five

were assigned intermediate or BV-positive scores. Interestingly, the healthy CST III-INERS samples contained the highest concentrations of lactic acid and had the lowest pH values within CST III-INERS and are observed clustering in the lower right with the CST I-CRISP samples in the lactic acid vs pH plot (**Fig 9**).

Variations in epithelial cell shedding rate, protein concentration and osmolality do not correlate with specific vaginal CSTs

In the previous chapter I presented a model for how vaginal epithelial shedding could select for specific species of bacteria. As an approximation of the shedding rate we estimated the total number of epithelial cells in the CVF samples. Despite a previous report that BV individuals shed epithelial cells at a higher rate (Gilbert et al., 2013), we observed no difference in the total number of epithelial cells per CVF sample across the different communities (**Fig 11**). Importantly, Gilbert et al. measured the number of cells per field of view and did not take into account variation in the volume of CVF. It should also be noted that our analysis was limited given a large number of samples with too low of a volume to perform the analysis.

CVF protein concentration is a common normalization tool for comparing CVF from different donors, however, to our knowledge it has not been determined if protein concentration varies with bacterial community. We saw no significant difference in mean protein concentration between the different bacterial communities with a mean concentration of 2.8 ± 1.8 g/L across all the samples (**Fig 12**). Similarly, no significant difference was observed in the mean osmolality for the different bacterial communities, with a mean value of 450 ± 68 mOsm (**Fig 13**), however, the range of osmolality extended much higher in CST IV-POLY samples

than in CST I-CRISP or CST III-INERS. Osmolality measurements in CST III-INERS samples may in fact represent a trend toward increased osmolality for this CST, however, with the limited number of samples the difference between the osmolality of CST I-CRISP CVF samples and CST III-INERS CVF samples was not significant.

Discussion

These results strongly suggest that the risk for STI acquisition associated with a polymicrobial community or a predominant *Lactobacilli* spp. that does not produce H_2O_2 can be explained by low levels of lactic acid. Across the 35 individuals we sampled, I observed the lowest acidity in CVF assigned to the polymicrobial CST IV-POLY, the highest acidity in CVF assigned to CST I-CRISP, characterized by H_2O_2 producer *L. crispatus*, and intermediate acidity in CVF assigned to CST II-INERS, characterized by *L. iners* which does not produce H_2O_2. Only the acidity of CVF obtained from women with CST I-CRISP was similar to the range of acidity that we and others have shown can inactivate HIV, *C. trachomatis* and HSV (Aldunate et al., 2013; Conti et al., 2009; Gong et al., 2014). Only the most acidic CVF samples observed in CST I-INERS and CST IV-POLY may be protective for BV-associated bacteria given our previous *in vitro* data (O'Hanlon et al., 2011). Even this is unlikely, however, as in the previous chapter I reported less inactivation of BV-bacteria by more acidic samples *ex vivo* than what was demonstrated *in vitro*.

The range of the pH in CST IV-POLY and CST I-CRISP did not overlap, and only one CST I-CRISP sample had a lactic acid concentration that overlapped with the range of CST IV-POLY. Given that the protections are thought to be due to a high lactic acid concentration and low pH, it is unlikely that a woman with a polymicrobial community would have the benefits that lactic acid can provide. The converse of this, however, that a community dominated by *Lactobacilli* spp. is always protective is not true. Three of the CST III-INERS samples were in the same acidity range as CST I-CRISP and three were not. Therefore, although Lactobacilli are likely necessary to reach high enough concentrations of lactic acid to provide protection, not all *Lactobacilli* spp.

are capable of sufficient acidification. Furthermore, our data suggest more variability among strains of *L. iners*, some of which acidify more and would be expected to be more protective than others. Samples that received a Nugent score of 4-10 were not restricted to CST IV-POLY. Instead, the same five CST-INERS samples that were less acidic were also given elevated Nugent scores. Inspecting these slides again revealed that the morphology and density of these *L. iners* strains was more similar to the morphology of BV-associated bacteria than other *Lactobacilli* spp. We can conclude that most women with a Nugent score of 4-10 have a vaginal bacterial community that fits CST IV-POLY. However, a Nugent score of 4-10 likely contains a small subset of women that fall into CST III-INERS. Furthermore, although Nugent score may not be reliable in distinguishing between CST IV-POLY and CST III-INERS, it may be adequate to predict risk, as the high Nugent score CST III-INERS samples were the least acidic.

Interestingly, Mirmonsef et al., who also observed higher pH values in CST III-INERS than in CST I-CRISP, demonstrated that the glycogen concentration in the CVF is highest for CST I-CRISP, lowest for CST IV-POLY and intermediate for CST III-INERS (2014). Although the authors hypothesized that glycogen levels may influence which strains of bacteria can colonize the vagina, it is also possible that high glycogen levels reflect a slow growing population of bacteria with a low metabolic demand. Taking into account the findings of the second chapter of this thesis, which demonstrate that Lactobacilli slow their growth rate in a more acidic environment, we would expect CST I-CRISP bacteria to grow slower than CST III-INERS bacteria, given the disparate pH.

Although lactic acid has long been thought to originate from Lactobacilli, little information has been provided to show the amount of lactic acid and the degree of acidity in

the absence of lactobacilli. Interestingly, the concentration of lactic acid in CST IV-POLY CVF samples was roughly 50-fold higher than the normal range of lactic acid in serum (Mizock and Falk, 1992). This suggests that even in the absence of a predominant *Lactobacilli* spp. lactic acid is accumulating in the vagina. It is possible some lactic acid is produced by the bacteria within CST IV-POLY, however, it is also possible that the lactic acid comes from the host epithelium. This may reflect a higher rate of anaerobic metabolism in the epithelium, given the hypoxic environment of the vagina.

Given that high vaginal concentrations of lactic acid and a low vaginal pH can effectively prevent the growth of BV-bacteria, these results also illuminate a possible mechanism by which BV bacteria could succeed in colonizing a Lactobacilli dominated vagina. Vaginal lactic acid is sufficient to inactivate BV-bacteria, however, even Lactobacilli are eventually inactivated by a high enough lactic acid concentration and low enough pH. Lactobacilli that acidify the vagina to a similar pH would be unable to inactive the other bacteria through the production of lactic acid alone. Given the wide range of pH and lactic acid concentrations associated with CST III-INERS it remains plausible that this CST could represent a transition point between a Lactobacilli dominated community and a polymicrobial community. This idea is in agreement with Gajer et al. which reported that transitions between a Lactobacilli dominated community and a polymicrobial community are more commonly from *L. iners* than from the other Lactobacilli species (2012). Furthermore, the only *Lactobacilli* spp. typically present in a CST IV-POLY is *L. iners.*

The osmolality of CVF is surprisingly higher than the normal range maintained in the body 270-300 mOsm/kg. Although an explanation for this remains unclear, it was true in each

CST. The increased concentration of bacteria observed in polymicrobial communities may cause a higher concentration of bacterial metabolites. Interestingly, the highest osmolarities were observed in CST IV-POLY. A high CVF osmolality may be responsible for watery discharge, which is a historic symptom of BV (Amsel et al., 1983). Not all women with BV report vaginal discharge as a symptom, which may reflect the low number of these samples in our dataset. Although such differences may be explained by different bacterial strains, no specific association was observed.

Chapter figures

Figure 7—CST assignments and heatmap of 35 CVF samples

Heatmap of log representation of each bacterial species within a CVF sample. Each horizontal line represents a different donor. Donors are ordered by CST, indicated by the CST assignments on the left.

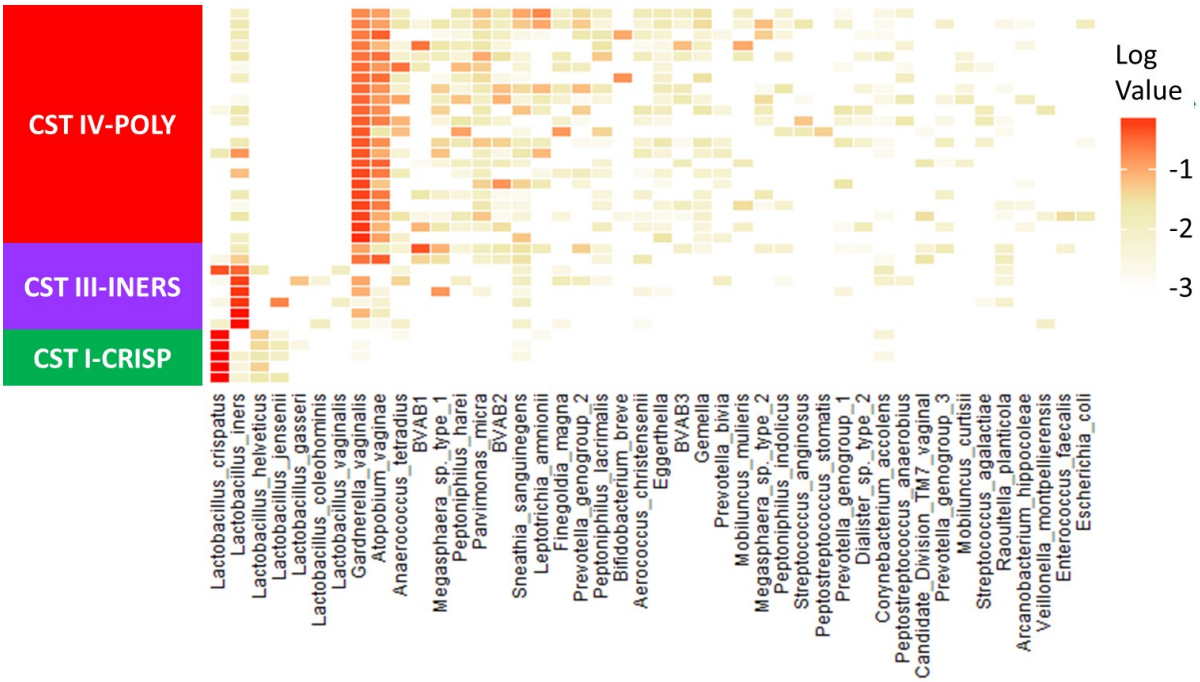


Figure 8—Measured pH across different CSTs

The pH under standard laboratory conditions (non-hypoxic) (**A**) and lactic acid concentrations (**B**) of the CVF samples organized by CST. Solid horizontal lines indicate the means of the populations.

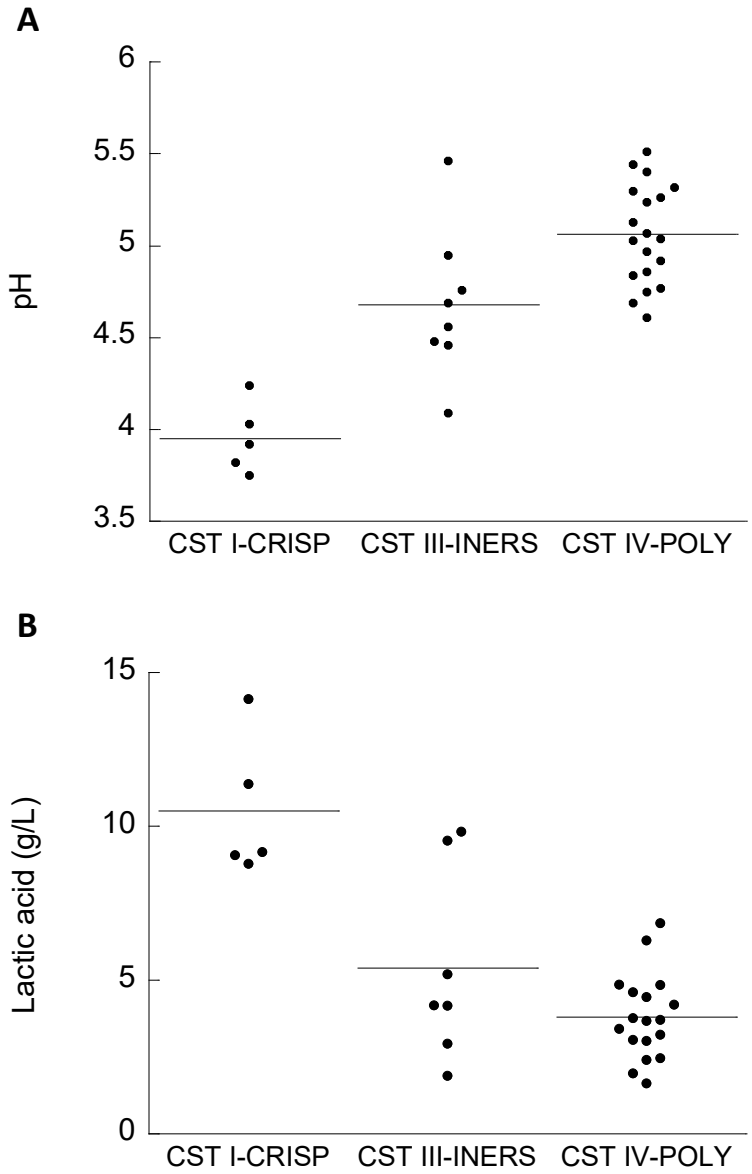


Figure 9—The lactic acid and pH of CVF samples correlates across all CSTs

The pH of the CVF samples plotted as a function of the lactic acid concentration and organized by CST.

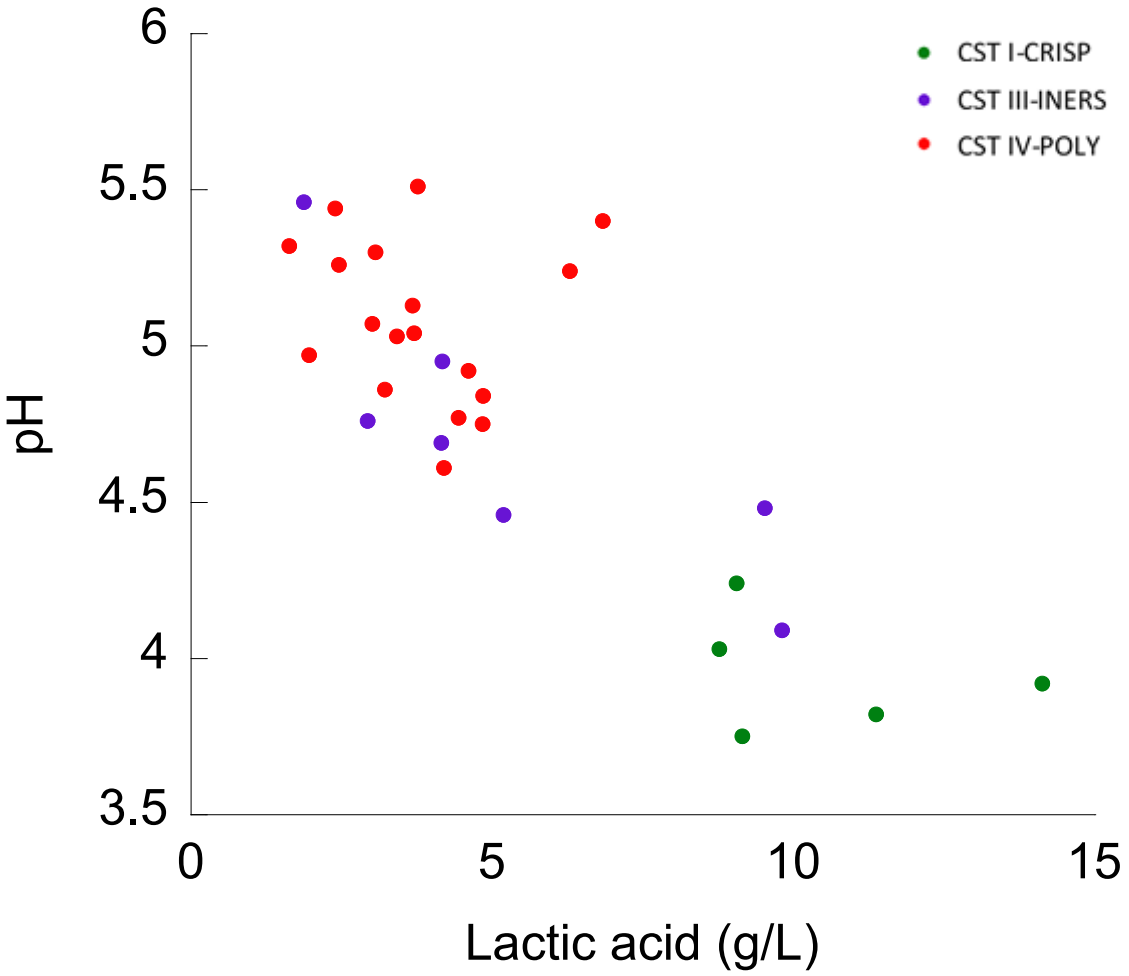


Figure 10—Nugent scoring can identify CST IV-POLY and CST I-CRISP but not CST III-INERS
Nugent scores assigned to the samples, organized by CST.

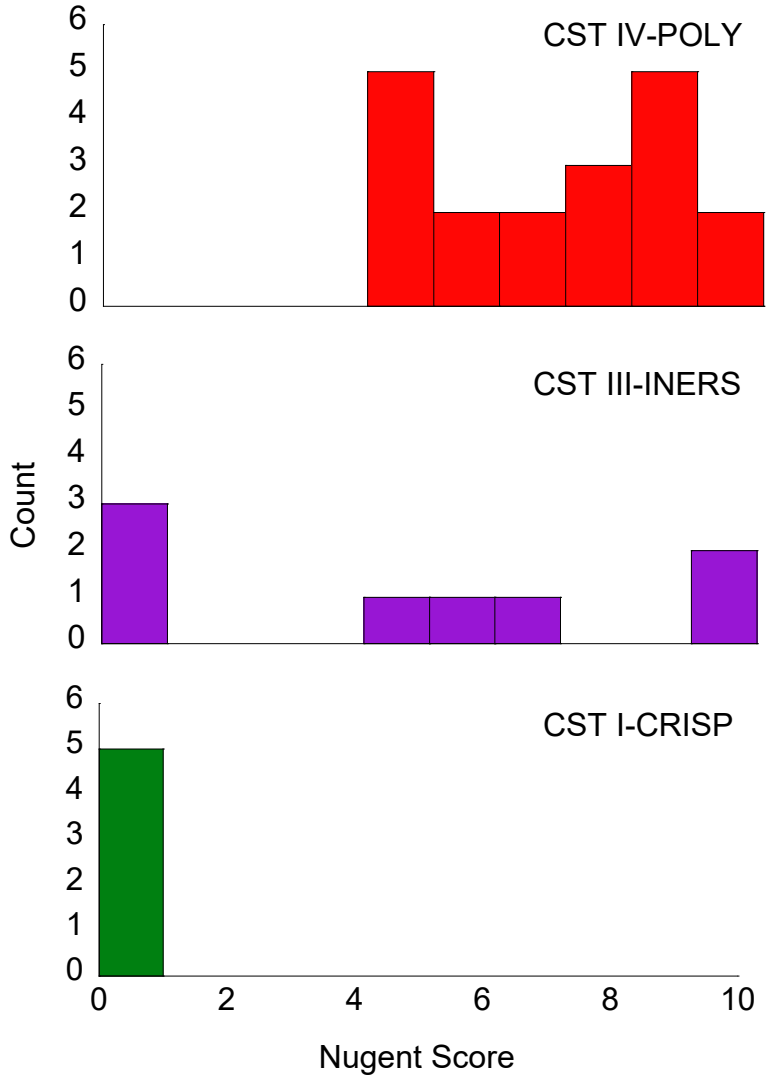


Figure 11—The average number of epithelial cells in CVF does not vary with CST
The estimated total number of epithelial cells per CVF sample organized by CST. Solid horizontal lines indicate the means of the populations, but note the apparently smalller range in CST I-CRISP.

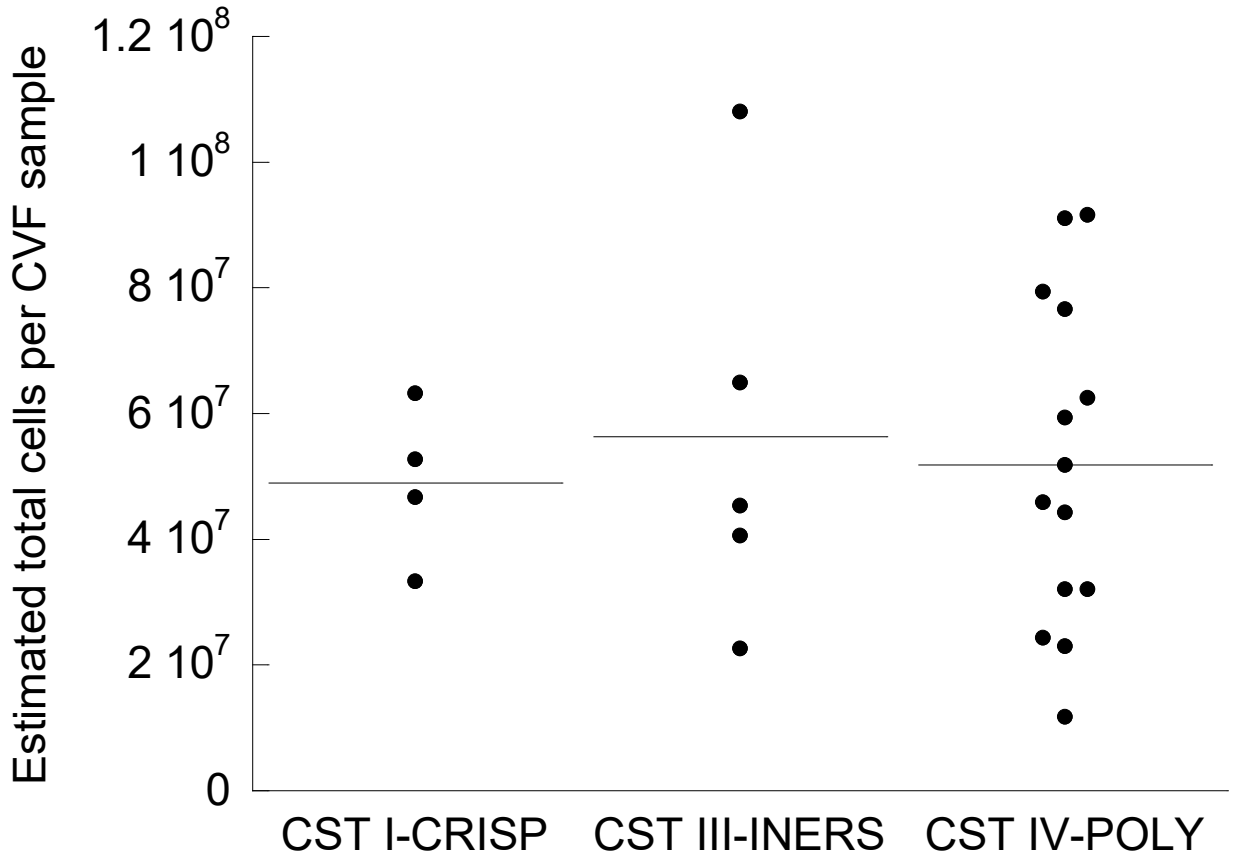


Figure 12—The average protein concentration of CVF does not vary with CST

The protein concentration of CVF samples organized by CST. Solid horizontal lines indicate the means of the populations. Again note the smaller range in the CST I-CRISP.

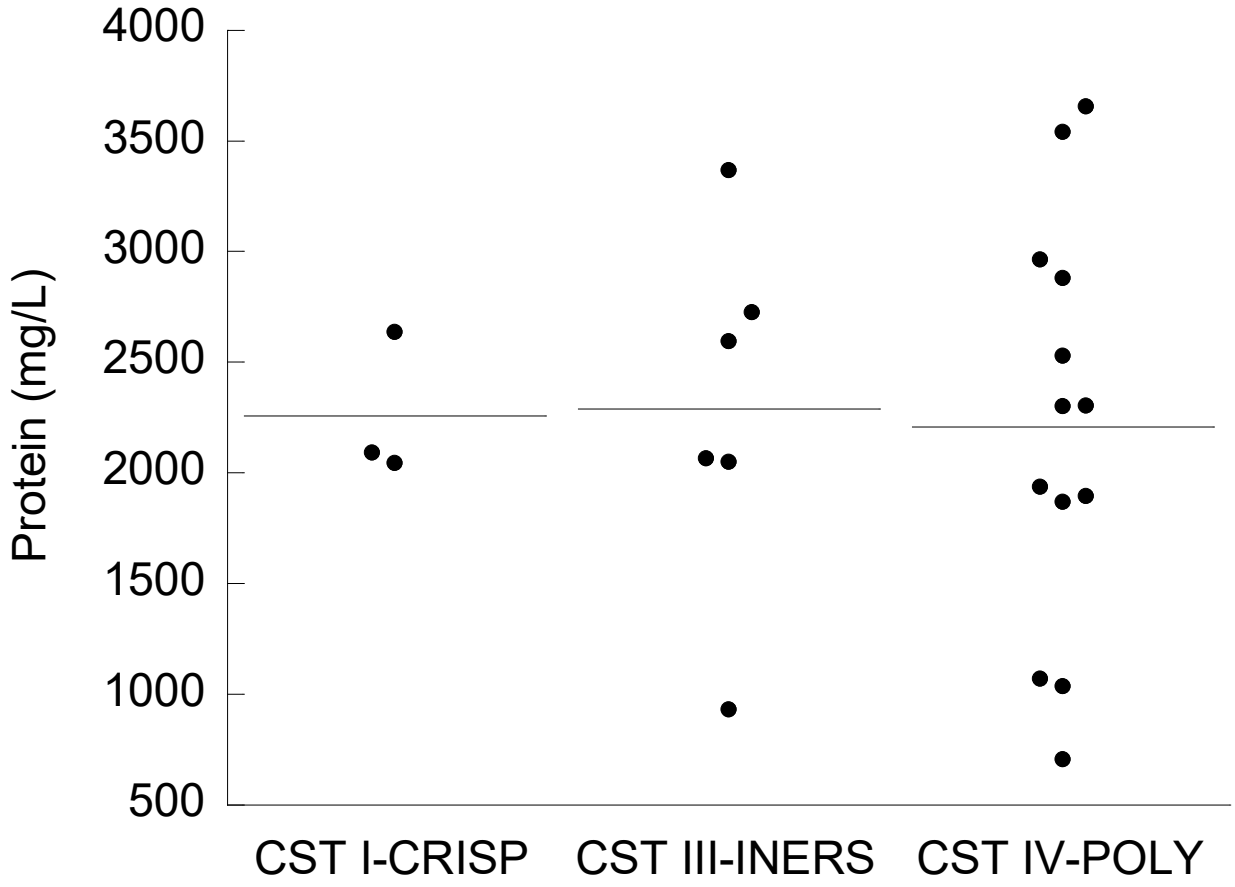
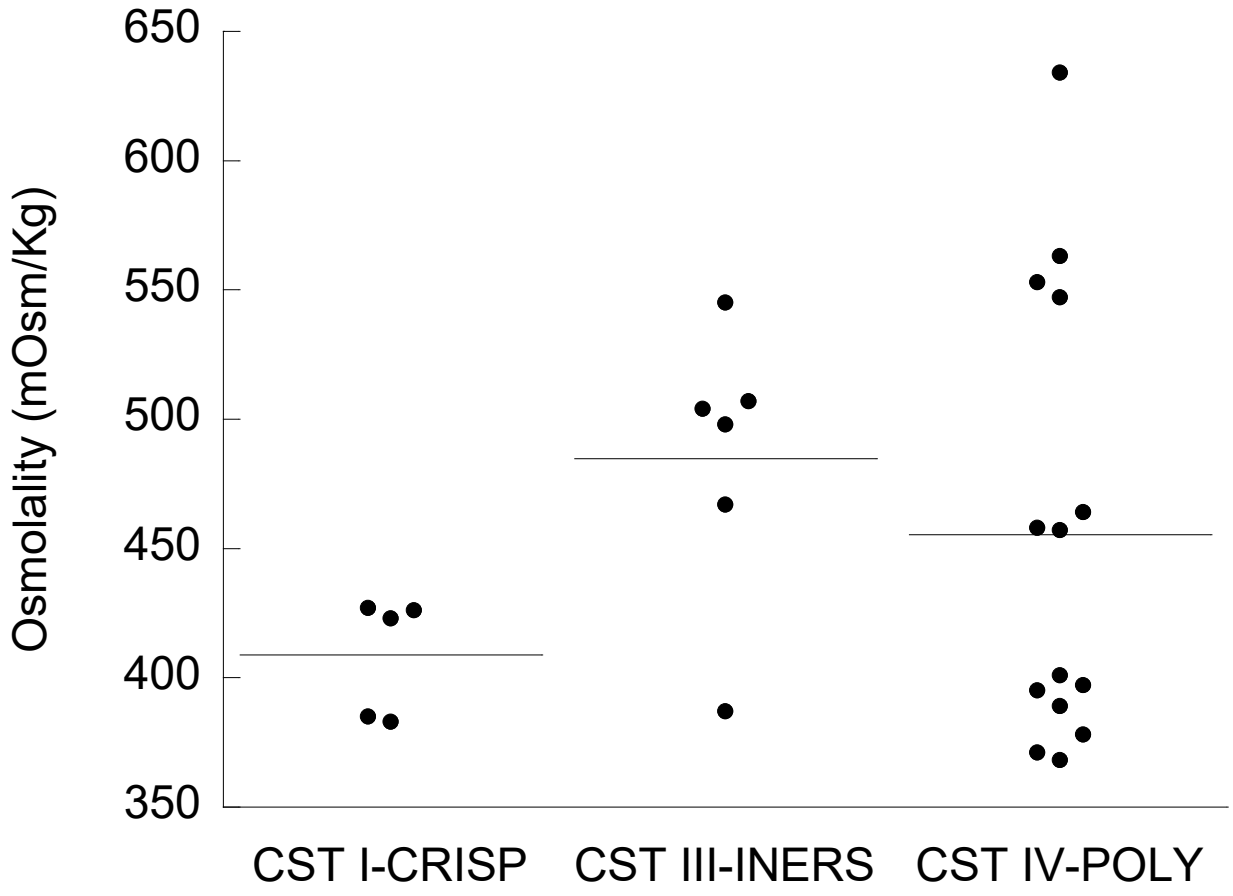


Figure 13—The average osmolality of CVF does not vary with CST

The osmolality of CVF samples organized by CST. Solid horizontal lines indicate the means of the populations. Note that in all three communities the CVF was markedly hyperosmolal with respect to serum (290-300 mOsm/Kg)



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Host antibody coating of vaginal bacteria and its implications

Chapter abstract

Gut bacteria are labeled with IgA and IgG antibodies, but to date, few attempts have been made to identify antibody labeling of vaginal bacteria, despite the increasing importance of these antibodies in maintaining gut bacterial communities. In these experiments I demonstrate that vaginal Lactobacilli are labeled by both IgG and IgA host antibodies using an immunofluorescent approach. Quantification of the fluorescent intensity was performed using image analysis of microscopic images, before successfully using flow cytometry to analyze the labeling. Both approaches reveal that the majority of vaginal Lactobacilli are labeled with host IgG and IgA and that in the majority of samples IgA labels more intensely than IgG. Although these experiments do not prove that the labeling is highly specific, a small subset of cultured bacteria from CVF could be recoated with dilute CVF supernatant, and had similar fluorescent intensity after staining. These experiments reveal a novel feature of vaginal bacteria that makes them more similar to gut bacteria than previously thought, and demonstrate the usefulness of flow cytometry in answering these questions.

Background Information

Gut surveillance of bacteria

Surveillance of gut bacteria is carried out primarily by Peyer's Patches, which are part of the Gut Associated Lymphoid Tissue (GALT) (Hamada et al., 2002; Mowat, 2003). Within the Peyer's Patches, M-cells, specialized intestinal epithelium cells, translocate bacteria from the lumen and deliver them to Antigen Presenting Cells (APCs). These APCs, specifically the motile Dendritic Cells (DCs), carry the bacteria to the mesenteric lymph nodes. The DCs then activate a number of other immune cells including B-cells. Activated B-cells home back to the mucosa and secrete IgA (Craig and Cebra, 1971), which is translocated back across the epithelium into the lumen.

B-cell homing and antibody secretion

Bacteria that are translocated by M-cells are confined to the mesenteric lymph nodes, and activated B-cells only home primarily to the mucosa. In this way the immune response is not systemic, and only results in the secretion of IgA across the mucus membrane (Johansen, 2005), preventing antibody secretion in the serum (Craig and Cebra, 1971). B-cells do not only home back to the gut, although a comprehensive list of other sites along the mucosa, such as the vagina, has not been established. In both rats and humans it has been shown that a nasal immune response leads to B-cell homing to the vagina (Pierce and Gowans, 1975), or antigen specific IgA secretion (Johansen, 2005). Furthermore, gut inoculation has led to the secretion of IgA and IgG into the respiratory tract of mice (Zuercher et al., 2002).

The function of antibodies in mucosal immunity

The function of IgA is complex (Macpherson et al., 2008; Mantis et al., 2011). Gut bacteria are extensively labeled by IgA and IgG, however, IgA appears to preferentially label commensal bacteria and may lead to pathologies such as Crohn's disease or irritable bowel syndrome when it labels pathogenic bacteria instead (Palm et al., 2014). The benefits of labeling are thought to include preventing the bacteria from crossing the intestinal epithelium, preventing bacteria from activating immune cells and potentially providing labeled bacteria a selective advantage. Specifically it has been shown that IgA labeling of bacteria enhances their ability to form biofilms and adhere to an epithelium *in vitro* (Bollinger et al., 2006).

It is possible that antibodies in the gut prevent bacteria from crossing the intestinal epithelium by trapping the bacteria in the mucus. This has been observed in the vagina with sperm (Castle et al., 1997), HSV and in the mouse gut with *Salmonella thyphimurium* (Wang et al., 2014). In these experiments, the presence of specific antibodies tethers their target to the mucus. The exact mechanism behind this is unclear, but it is likely mediated by weak transient reversible bonds between antibody F_c regions and mucin fibers.

Despite immunoglobulin being the second most concentrated protein within CVF (Panicker et al., 2010), it is unknown if it plays any role in the dynamics of vaginal bacterial communities. The high concentration of immunoglobulin potentially originates from the blood serum via interstitial fluid. This is supported by a similar 1:5 IgA to IgG ratio observed in both vaginal mucus and serum (Chipperfield and Evans, 1975; Gonzalez-Quintela et al., 2007; Wang et al., 2014), however, the total concentration of antibody in CVF is also about 50-fold lower

than in serum. The other possibility is that the immunoglobulins are secreted by immune cells that have homed to the vagina. One could imagine how the presence of certain gut bacteria could affect the survival of certain species in the vagina. If secreted IgA label a subset of the vaginal bacteria it could promote the survival of these species by allowing them to avoid detection by the immune system, or in the case of *G. vaginalis* could prove instrumental in its ability to form a biofilm.

Non-vaginal strain oral probiotics support vaginal Lactobacilli

Probiotic approaches to treat BV have had mixed success (Falagas et al., 2007; Homayouni et al., 2014; Senok et al., 2009). There is no evidence that oral probiotics provide long-term treatment of BV, however, it seems there is a short-term change in the composition of the vaginal microbiota (Macklaim et al., 2015; Reid et al., 2003; Vujic et al., 2013). Although this is likely not efficacious for the treatment of BV, it may provide clues as to how a vaginal microbiota is maintained by the host. Specifically, there is evidence that oral administration of non-vaginal *Lactobacilli* spp. leads to an increase in the concentration of Lactobacilli within the vagina (Macklaim et al., 2015). The vaginal tract is not connected to the digestive tract, and the evidence to date has failed to detect significant amounts of gut bacteria in the vagina. Therefore, it is not surprising that the *Lactobacilli* spp. within the probiotic are not the species that appear within the vagina. Instead, vaginal *Lactobacilli* spp., usually *L. iners*, become more abundant. This suggests a connection between bacteria present in the gut, and the permissibility of the vagina to specific strains of bacteria. It is possible that this connection is maintained by the mucosal immune system.

Research Goals

I: Can host IgA or IgG antibodies be identified on vaginal bacteria?

The vaginal immunoglobulins are either being secreted into the CVF by immune cells, or already present in the interstitial fluid that is deposited into the vagina. In either case the high concentration of immunoglobulins may coat vaginal bacteria. Given the importance of antibody coating bacteria in the gut, it was paramount to establish whether vaginal bacteria are coated as well. Boskey reported pilot observations that suggested vaginal bacteria are coated in the appendix of her Biophysics PhD thesis (2000). To confirm and quantify her pilot observations, I used both a microscopic and flow cytometry to detect and quantify antibodies on the surfaces of *ex vivo* vaginal bacteria.

II: Can vaginal bacteria be cultured and recoated with host antibody?

The observation of host antibodies on vaginal bacteria cannot confirm the specificity of the antibodies to the bacteria. Understanding if the antibody binding is specific is important for understanding how the antibodies might affect the vaginal community, as well as where they are originating. To approach this question I used cultured antibody free vaginal bacteria from CVF samples, and attempted to recoat the bacteria with supernatant from the original CVF sample.

Materials & Methods

CVF collection and processing

All CVF samples were self-collected by donors by inserting and removing an Instead® SoftCup menstrual collection device (Boskey et al., 2003). Softcups were placed in 50 mL conical tubes and centrifuged to collect the CVF. A slide was made for each sample to assign a Nugent score. For microscopy experiments CVF was mixed with FITC conjugated 0.5 µm diameter fiduciary beads before being pipetted onto a glass slide and allowed to briefly dry before being washed three times with PBS. For flow cytometry experiments, CVF diluted 1:10 was passed spun through a 10 µm cell strainer to remove epithelial and immune cells. The filtered bacteria were pelleted by centrifugation and washed three times with PBS.

For recoating experiments an aliquot of the original CVF sample diluted 1:10 in saline was centrifuged and the collected supernatant was sterile filtered using 0.2 µm spin filters. Sterile dilute supernatants were stored at -80°C before being thawed for recoating experiments.

Antibody labeling

Alexa488 conjugated Goat anti-human IgA, IgG or IgM F(AB)s were diluted in PBS 1:400 and incubated with *ex vivo* unfixed bacteria either mounted on slides for microscopy, or in solution for flow cytometry. F(AB) incubations were at room temperature and lasted 30 minutes. Afterward PBS washes were applied directly to the slide, or the bacteria in solution

were rinsed by spinning down, aspirating and resuspending in PBS three times. Hoechst solution was added to the bacteria on the slides to stain for DNA.

Microscopy and quantification

Bacteria were visualized using the Zeiss Axiovert 200 inverted lighted microscope, and images were recorded at 100x magnification. Bacteria were identified by DNA staining, and fluorescent intensity measurements of individual bacteria were made in ImageJ. The fiduciary beads that were mounted on the slide along with the bacteria, were used to normalize fluorescent intensity measurements.

Flow cytometry

Flow cytometry was performed on the BD FACSCanto using BD FACSDiva software. Before running bacterial solutions on the instrument, samples were pipetted over a 35 µm cell strainer. Bacterial populations were identified and gated based on their location in forward scatter and side scatter area plots. The FITC intensity of at least 1000 gated events was recorded per run. The instrument was calibrated using FITC conjugated beads to allow for fluorescent intensity comparisons between different experiments. FCS files were converted into CSV files and data manipulation and graphing was performed in R.

Bacteria culturing and recoating

To obtain antibody free bacteria, CVF samples were plated on Brucella broth, 5% sheep blood plates. Blood plates were incubated at 37°C in anaerobic jars with oxygen scavenger

sachets. After 2-3 days of incubation, bacterial colonies were scraped off the plate, vigorously resuspended in saline, and rinsed three times with PBS.

To recoat cultured bacteria with antibody, the bacteria were incubated in suspension with either the sterile filtered dilute supernatant obtained from the original CVF sample or PBS for one hour at room temperature. After incubation the bacteria were rinsed three times in PBS, before being incubated with fluorescently conjugated F(AB)s as described above.

Results

Host antibodies can be visualized on vaginal Lactobacilli

Given the high concentration of Immunoglobulins (Ig) in the gut I wanted to determine if vaginal bacteria were labeled by these host antibodies. Utilizing a microscopy approach I treated CVF samples obtained from women with a Nugent score less than 4 with fluorescently labeled anti-human Ig F(AB). Anti-human Ig F(AB) was used instead of complete Igs to rule out the possibility of the secondary antibodies being recognized by Fc-receptors on the bacteria. In all samples tested I observed significant labeling of the vaginal bacteria with both F(AB) anti-IgG and F(AB) anti-IgA, confirming the presence of host IgG and IgA antibodies on the bacteria (**Fig 14**). F(AB) anti-IgM had very limited labeling compared to the other two. For most donors the majority of bacteria appeared to be labeled with IgA and IgG (data not shown). Individual bacterial fluorescence was quantified by measuring the fluorescence of each bacteria and normalized to fiduciary beads imaged along with the bacteria. In all but one of the samples the staining of anti-IgA exceeded that of anti-IgG, despite IgG being more concentrated than IgA in CVF (**Fig 15**). To verify that bacteria could not bind F(AB)s through an unknown mechanism, I also labeled the bacteria with anti-Mouse IgG. No significant staining was observed (data not shown). I also used the microscopy based approach to detect whether antibodies coat bacteria in BV samples, but the results were inconclusive due to the complex biofilms that BV bacteria form.

Flow Cytometry can be used to quantify coating by host antibodies

Due to both the cumbersome nature of microscopy based quantification as well as the limited number of bacteria included in the analysis, I adapted flow cytometry protocols to better quantify the degree of host antibody labeling in CVF samples. Samples were prepared similarly as to the microscopy experiments, and then analyzed by flow cytometry to measure the fluorescence of individual bacteria. Significant labeling by anti-IgA and anti-IgG was observed in nearly all 5 samples that vastly exceeded the labeling of anti-IgM (**Fig 16**). Nearly all of the bacteria were labeled by IgA and/or IgG, as compared to the fluorescent intensities of control, unlabeled bacteria. In 4 of the 5 samples the median fluorescent intensity of bacteria labeled with anti-IgA labeled was higher than anti-IgG (**Table 1**). Although in samples such as HA08 and HA14, clear bimodal distributions could be observed and are not easily described by a median measurement. Overall, the flow cytometry based approach greatly corroborated the microscopy based approach, confirming the presence of host antibody labeling of vaginal Lactobacilli, and validating its use as a tool.

Vaginal bacteria can be recoated by host antibodies after being grown in culture

The presence of host antibodies on vaginal bacteria could be due to specific antibody recognition of the vaginal bacteria, or nonspecific given the long period of time the bacteria spend exposed to a high concentration of antibody. To rule out this possibility I obtained host derived antibody free bacteria, by culturing CVF samples on agar plates, and resuspending the colonies in saline. Although a vaginal bacterial community predominated by *Lactobacilli* spp. tends to be monomicrobial, there could still be intrastrain variability. This approach may

overestimate the presence of fast growing bacteria compared to slow growing bacteria in the final solution, but it should also preserve some of the community diversity.

Cultured bacteria were treated with either PBS, or the supernatant obtained from the original CVF sample, diluted 10-fold. PBS treatment did not result in staining. Only in the supernatant treated sample did anti-IgA and anti-IgG label the bacteria (**Fig 17**), however, only a fraction of the cultured bacteria were labeled, unlike the *ex vivo* uncultured bacteria. For one of the samples (K001) IgA and IgG labeled with very similar intensity to that of the uncultured bacteria, and for the other (HA04) only the brightest labeling returned (**Fig 16**). Despite variable staining, microscopic inspection of the cultured bacteria revealed a homogenous community of gram-positive bacteria, consistent with a monomicrobial population.

Discussion

Here I report that vaginal Lactobacilli are bound by host antibodies, similar to the bacteria in the gut. Nearly all of the vaginal Lactobacilli stained positive for host IgA and IgG if analyzed *ex vivo*, however, only a portion of the Lactobacilli that were cultured stained positive after exposure to CVF supernatant. Although microscopy revealed homogenous morphology amongst the stained bacteria, there could still be variation that leads to the expression of specific epitopes in a subset of bacteria that grows faster on the agar plates than the vaginally dominate strain.

Regardless of the reduced staining, a population of cultured bacteria could be recoated by the dilute supernatant of the original CVF sample. Although one might attribute a reduced number of labeled bacteria to the reduced concentration of antibodies, the signal in cultured bacteria is similarly intense to the uncultured bacteria. This suggests the presence a population of antibodies within the CVF that specifically bind one or more vaginal strains of Lactobacilli. However, it is also possible that binding is mediated through some other mechanism such as F_c-receptors, although, this would require only a subset of the vaginal bacteria to have F_c-receptors. Furthermore, staining for IgA provided the most intense signal compared to IgG and IgM in most samples, despite IgA being significantly less concentrated than IgG in CVF (Chipperfield and Evans, 1975; Gonzalez-Quintela et al., 2007; Wang et al., 2014). If the binding was nonspecific one would expect labeling to correlate with the concentrations of the antibodies.

The origin of the antibodies that label vaginal bacteria is still uncertain. Despite a lack of organized immune tissue in the vagina the bacteria might be surveyed by the immune system, leading to antibody secretion that is specific to the vaginal bacteria. Given the effect of an oral Lactobacilli probiotic on the vaginal bacterial community, another possibility is that the immune system detects bacteria at mucosal sites other than the vagina, but leads to antibody secretion in multiple sites, including the vagina. Even if the bacteria detected by the immune system were not vaginal strains of bacteria, closely related or similar bacteria could lead to the eventual secretion of antibodies that label vaginal bacteria. It is also possible that local surveillance and cross talk between body sites are dependent on the current vaginal bacterial community. Vaginal Lactobacilli *L. crispatus* and *L. jensenii* have been described as non-inflammatory to the vaginal epithelium, and through lactic acid production may in fact reduce inflammation (Aldunate et al., 2015), and limit the possibility of immune surveillance when these bacteria are present.

The discovery that vaginal bacteria are labeled by host antibodies is an important step to understanding how the host immune system may play a role in determining a vaginal bacterial community.

Chapter figures

Figure 14—Immunofluorescence reveals IgA and IgG staining of vaginal Lactobacilli
Labeling of *ex vivo* vaginal Lactobacilli obtained from a CVF sample with a Nugent score less than 4 with anti-human IgA (top) and anti-human IgG (bottom). DNA staining reveals individual bacteria. Scale bar = 10 µm

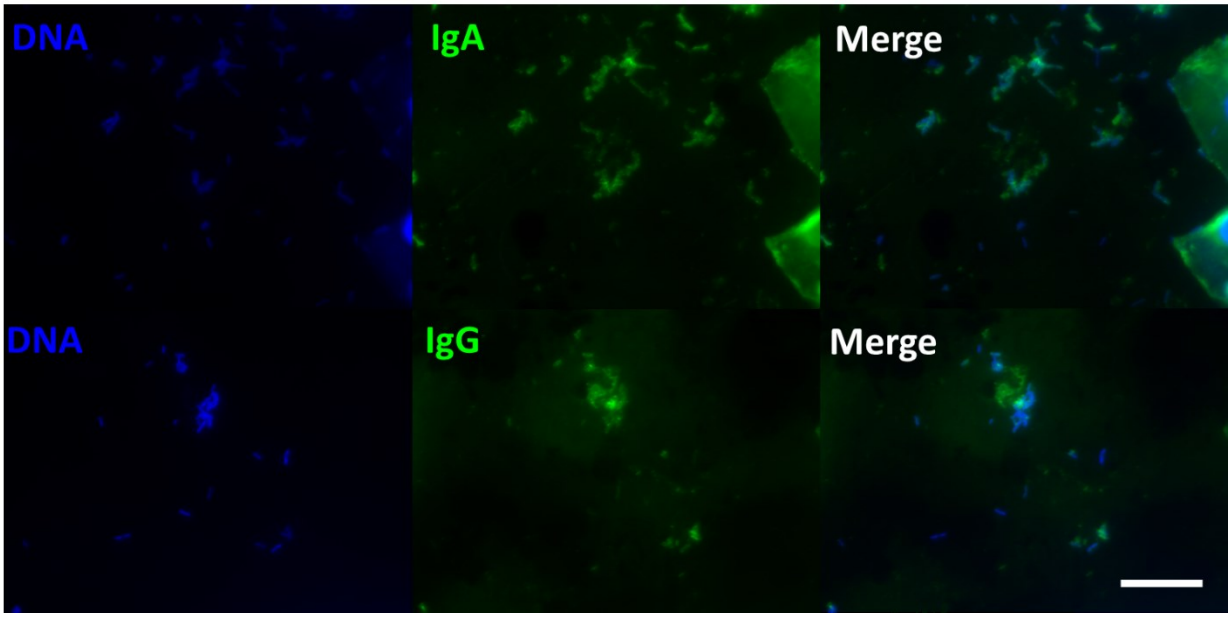


Figure 15—Fluorescent intensity distributions of antibody coated bacteria using image based quantification

Microscopy based quantification of fluorescence intensity of individual bacteria labeled with fluorescently labeled anti-human IgA, IgG or IgM (A, G, M) in 6 different CVF samples with Nugent score 0-3. The intrinsic fluorescence was measured using unlabeled bacteria.

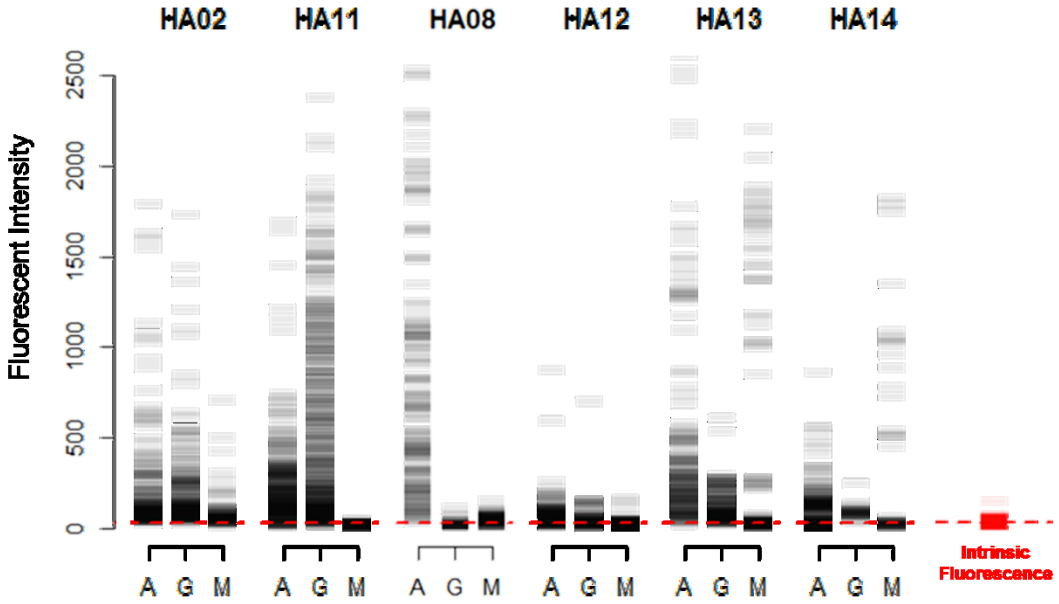


Figure 16— Fluorescent intensity distributions of antibody coated bacteria using flow cytometry

Flow cytometry based distribution of fluorescence intensity of individual bacteria labeled with fluorescently labeled anti-human IgA, IgG or IgM in 5 different CVF samples with Nugent score 0-3. Fluorescent intensities are normalized to FITC beads.

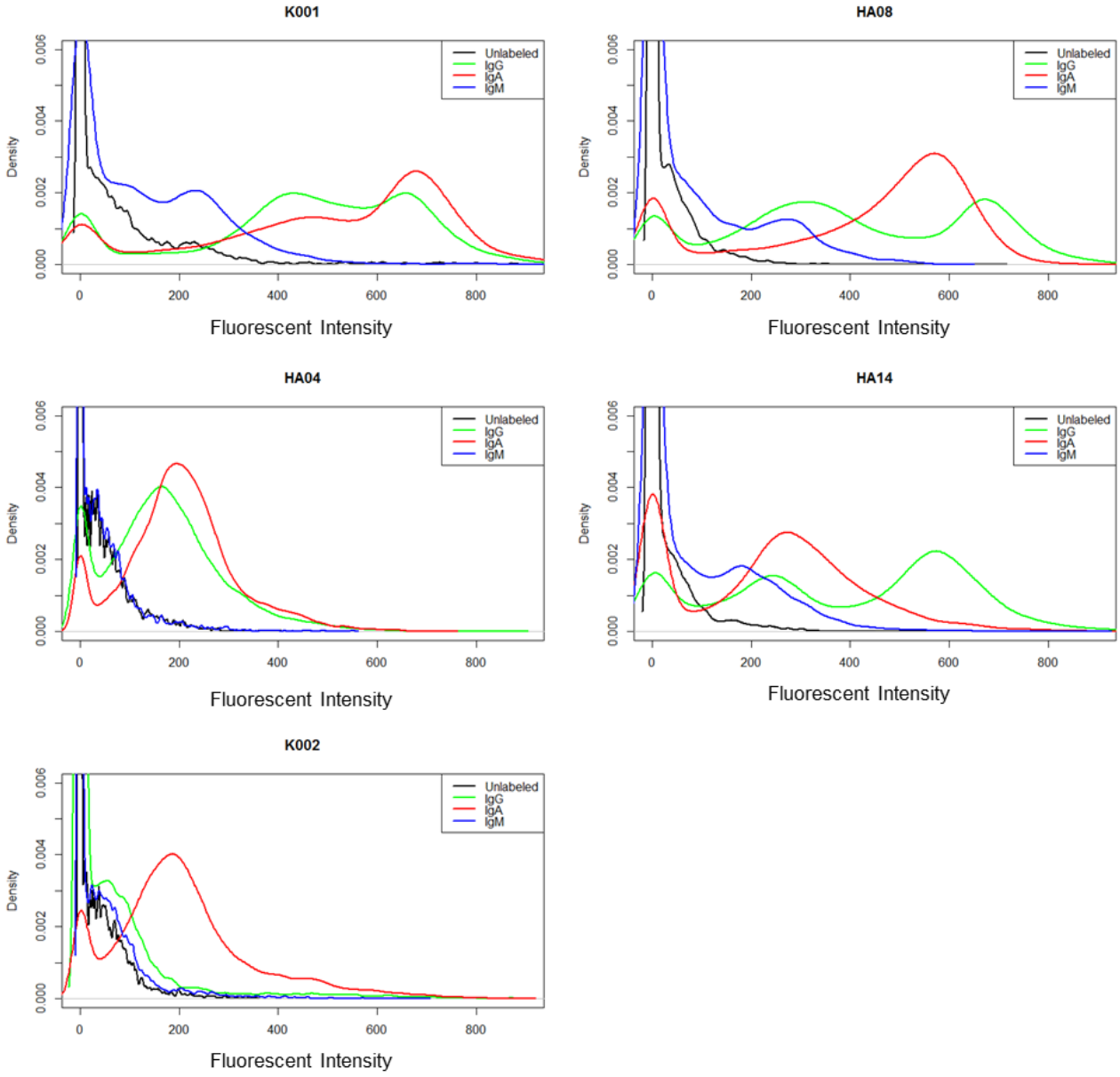
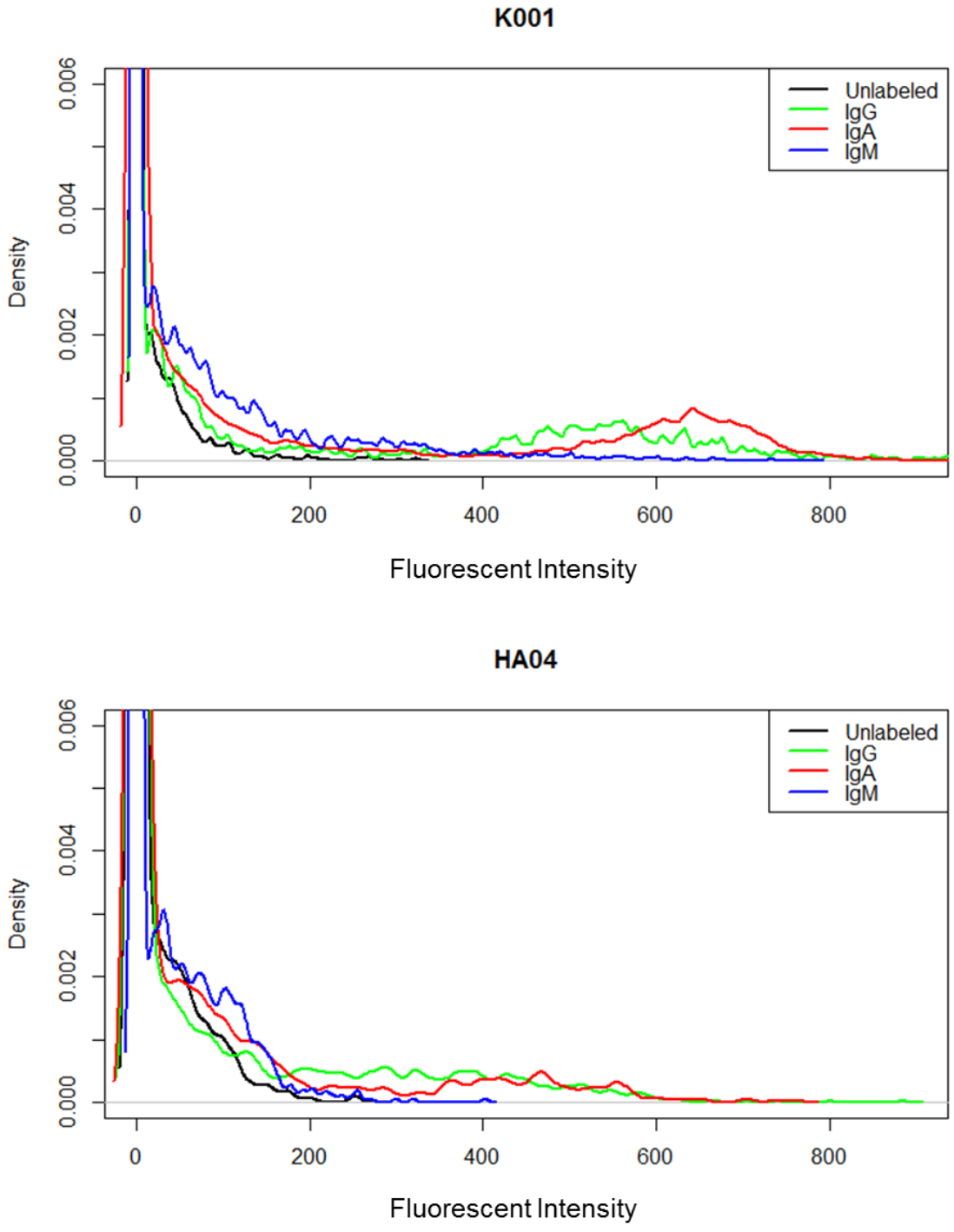


Figure 17—Cultured bacteria from CVF samples can be recoated with antibodies from CVF supernatant

Flow cytometry based distribution of fluorescence intensity of bacteria from CVF after incubation with supernatant from the original CVF, and labeled with anti-human IgA, IgG or IgM.



Chapter tables

Table 1—The median intensity of IgA staining tends to be higher than IgG or IgM
Median fluorescent intensity of bacteria analyzed by flow cytometry from 5 different donors and stained for IgG, IgA or IgM.

	Median Fluorescent Intensity			
Donor	**Unlabeled**	**IgG**	**IgA**	**IgM**
K001	0	483	559	94
HA04	0	161	199	0
HA08	0	371	502	24
HA14	0	417	253	26
K002	0	0	188	0

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